Methods for the Storage and Synthesis of Nucleic Acids using a Solid Support

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application No. 60/175,307, filed January 10, 2000, the disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of molecular biology. In particular, the present invention relates to the fields of storage, synthesis and amplification of nucleic acids. Specifically the invention relates to storage of RNA (particularly mRNA) on a solid matrix or support and to manipulation of the RNA by a number of molecular biology techniques including RT-PCR and cDNA synthesis (particularly cDNA library synthesis).

Related Art

Storage of Nucleic Acids

For many projects, generation of numerous DNA samples from biological specimens is routine. Handling and archiving a large collection can become a logistical problem for the laboratory. One solution, used in forensic labs, is the blood-storage medium FTA® Cards. The FTA® GeneCard is a chemically-treated filter paper designed for the collection and storage of biological samples for subsequent DNA analysis (1-3). It is suitable for storage of blood samples, as well as mammalian cells and tissues for PCR analysis and other genomic DNA applications (4). It is useful for recovery of plasmid DNA for PCR and transformation from archived bacterial cultures and colonies (5-6), as well as for storage and recovery of M13 phage for DNA sequencing applications (M. Goldsborough, personal communication).

An FTA® Card can be used to store genomic DNA in the form of dried spots of human whole blood, the cells of which were lysed on the paper.

Stored at room temperature, genomic DNA on FTA® paper is reported to be stable at least 7.5 years (Burgoyne, *et al.*, Conventional DNA Collection and Processing: Disposable Toothbrushes and FTA®Paper as a Non-threating Buccal-Cell Collection Kit Compatible with Automatable DNA Processing, 8th International Symposium on Human Identification, September 17-20, 1997). Before analysis of the captured DNA, a few simple washing steps remove the stabilizing chemicals and cellular inhibitors of enzymatic reactions. Since the DNA remains with the paper, the manipulations to purify the DNA are simplified and amenable to automation. DNA samples on FTA® Cards offer a very compact archival system compared to glass vials or plastic tubes located in precious freezer space. Storage of RNA on dry solid medium is also described (see Burgoyne, U.S. Patent No. 5,976,572).

Reverse Transcription of RNA

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA polymerases. All known reverse transcriptases require a primer to synthesize a DNA transcript from an RNA template. Historically, reverse transcriptase has been used primarily to transcribe mRNA into cDNA which can then be cloned into a vector for further manipulation.

Avian myoblastosis virus (AMV) reverse transcriptase was the first widely used RNA-dependent DNA polymerase (Verma, *Biochem. Biophys. Acta* 473:1(1977)). The enzyme has 5'→3' RNA-directed DNA polymerase activity, 5'→3' DNA-directed DNA polymerase activity, and RNase H activity. RNase H is a processive 5' and 3' ribonuclease specific for the RNA strand for RNA-DNA hybrids (Perbal, *A Practical Guide to Molecular Cloning,* New York: Wiley & Sons (1984)). Errors in transcription cannot be corrected by reverse transcriptase because known viral reverse transcriptases lack the 3'→5' exonuclease activity necessary for proofreading (Saunders and Saunders, *Microbial Genetics Applied to Biotechnology,* London: Croom Helm (1987)). A detailed study of the activity of AMV reverse transcriptase and its associated RNase H activity has been presented by Berger *et al.*, *Biochemistry* 22:2365-2372 (1983).

Another reverse transcriptase which is used extensively in molecular biology is reverse transcriptase originating from Moloney murine leukemia virus (M-MLV). *See, e.g.,* Gerard, G.R., *DNA* 5:271-279 (1986) and Kotewicz, M.L., *et al., Gene* 35:249-258 (1985). M-MLV reverse transcriptase substantially lacking in RNase H activity has also been described. *See, e.g., U.S.* Patent No. 5,244,797.

PCR Amplification of RNA

Reverse transcriptases have been extensively used in reverse transcribing RNA prior to PCR amplification. This method, often referred to as RNA-PCR or RT-PCR, is widely used for detection and quantitation of RNA.

To attempt to address the technical problems often associated with RT-PCR, a number of protocols have been developed taking into account the three basic steps of the procedure: (a) the denaturation of RNA and the hybridization of reverse primer; (b) the synthesis of cDNA; and (c) PCR amplification. In the so-called "uncoupled" RT-PCR procedure (*e.g.*, two-step RT-PCR), reverse transcription is performed as an independent step using the optimal buffer condition for reverse transcriptase activity. Following cDNA synthesis, the reaction is diluted to decrease $MgCl_2$ and deoxyribonucleoside triphosphate (dNTP) concentrations to conditions optimal for *Taq* DNA Polymerase activity, and PCR is carried out according to standard conditions (*see* U.S. Patent Nos. 4,683,195 and 4,683,202). In contrast, "coupled" RT-PCR methods use a common or compromised buffer for reverse transcriptase and *Taq* DNA Polymerase activities. In one version, the annealing of reverse primer is a separate step preceding the addition of enzymes, which are then added to the single reaction vessel. In another version, the reverse transcriptase activity is a component of the thermostable *Tth* DNA polymerase. Annealing and cDNA synthesis are performed in the presence of Mn++, then PCR is carried out in the presence of Mg++ after the removal of Mn++ by a chelating agent. Finally, the "continuous" method (*e.g.*, one-step RT-PCR) integrates the three RT-PCR steps into a single continuous reaction that avoids the opening of the reaction tube for component or enzyme addition. Continuous RT-PCR has been described as a single enzyme system using the reverse transcriptase activity of thermostable *Taq* DNA Polymerase and *Tth* polymerase and as a two-enzyme system using AMVRT and *Taq* DNA Polymerase wherein the initial 65°C RNA denaturation step was omitted.

cDNA and cDNA Libraries

In examining the structure and physiology of an organism, tissue or cell, it is often desirable to determine its genetic content. The genetic framework of an organism is encoded in the double-stranded sequence of nucleotide bases in the deoxyribonucleic acid (DNA) which is contained in the somatic and germ cells of the organism. The genetic content of a particular

segment of DNA, or gene, is only manifested upon production of the protein which the gene encodes. In order to produce a protein, a complementary copy of one strand of the DNA double helix (the "coding" strand) is produced by polymerase enzymes, resulting in a specific sequence of ribonucleic acid (RNA). This particular type of RNA, since it contains the genetic message from the DNA for production of a protein, is called messenger RNA (mRNA).

Within a given cell, tissue or organism, there exist myriad mRNA species, each encoding a separate and specific protein. This fact provides a powerful tool to investigators interested in studying genetic expression in a tissue or cell --mRNA molecules may be isolated and further manipulated by various molecular biological techniques, thereby allowing the elucidation of the full functional genetic content of a cell, tissue or organism.

One common approach to the study of gene expression is the production of complementary DNA (cDNA) clones. In this technique, the mRNA molecules from an organism are isolated from an extract of the cells or tissues of the organism. This isolation often employs solid chromatography matrices, such as cellulose or Sepharose, to which oligomers of thymidine (T) have been complexed. Since the 3' termini on all eukaryotic mRNA molecules contain a string of adenosine (A) bases, and since A binds to T, the mRNA molecules can be rapidly purified from other molecules and substances in the tissue or cell extract. From these purified mRNA molecules, cDNA copies may be made using an enzyme having reverse transcriptase (RT) activity, which results in the production of single-stranded cDNA molecules complementary to all or a portion of the mRNA templates. Incubating the single-stranded cDNA under appropriate conditions allows synthesis of double-stranded DNA which may then be inserted into a plasmid or a vector.

This entire process, from isolation of mRNA to insertion of the cDNA into a plasmid or vector to growth of host cell populations containing the isolated gene, is termed "cDNA cloning." If cDNAs are prepared from a number of different mRNAs, the resulting set of cDNAs is called a "cDNA library," an appropriate term since the set of cDNAs represents the different populations of functional genetic information (genes) present in the source cell, tissue or organism. Genotypic analysis of these cDNA libraries can yield

much information on the structure and function of the organisms from which they were derived.

In traditional production methods, the cDNA molecules must be size fractionated and multiple phenol/chloroform extractions and ethanol precipitations performed. Each of these requirements has inherent disadvantages, such as product loss and limitations in cDNA yield due to multiple extractions/precipitations (Lambert, K.N., and Williamson, V.M., *Nucl. Acids Res. 21(3):775-776 (1993)).*

These disadvantages have been partially addressed in the literature. For example, several investigators have reported methods for the isolation of polyA+ mRNA from cell and tissue samples by binding the mRNA to latex or paramagnetic beads coupled with oligo(dT); single-stranded cDNA molecules may then be produced by reverse transcription of these immobilized mRNA molecules (Lambert, K.N., and Williamson, V.M., *Nucl. Acids Res. 21(3):775-776 (1993);* Kuribayashi-Ohta, K., *et al., Biochim. Biophys. Acta 1156:204-212 (1993);* Sasaki, Y.F., *etal., Nucl. Acids Res. 22(6):987-992 (1994);* Mészáros, M., and Morton, D.B., *BioTechniques 20(3):413-419 (1996);* Fellman, F., *et al., BioTechniques 21(5):766-770 (1996)).* Such solid phase synthesis methods are less prone to the yield limitations resulting from the extraction/precipitation steps of the traditional methods.

However, these methods still have several important limitations. For example, each of these methods relies on PCR amplification prior to cloning of the cDNA molecules, often resulting in biased cDNA libraries (*i.e.*, highly expressed sequences predominate over those that are expressed in lower quantities). In addition, these methods often are less efficient than conventional cDNA synthesis methods which use solution hybridization of the primer-adapter to the template (*i.e.*, rotational diffusion is required for increased hybridization rates; *see* Schmitz, K.S., and Schurr, J.M., J. Phys. *Chem. 76:534-545 (1972);* Ness, J. V., and Hahn, W.E., *Nucl. Acids Res. 10(24):8061-8077 (1982)).* Finally, the above-described techniques use heat or chemical denaturation to release the nascent cDNA molecules from the solid phase for further processing, which can result in product loss and/or damage.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to a solid medium or support for use in the storage (preferably the long term storage) of nucleic acids (e.g., DNA and RNA, ribosomal RNA and messenger RNA), particularly polyA RNA or mRNA which comprise the use of this solid medium or support. In particular, the invention relates to a method for storage and transport of such nucleic acids on the solid medium, as well as to methods which involve either recovery of the nucleic acids from the solid medium, and/or the use or manipulation of the nucleic acids obtained from or contained by the solid medium. Such use or manipulation includes, for example, digestion (e.g., with one or more nucleases, exonucleases or endonucleases such as restriction enzymes), synthesis (e.g., with one or more polymerases and/or reverse transcriptases), amplification (e.g., by polymerase chain reaction with one or more polymerases), sequencing (e.g., with one or more polymerases), or transformation or transfection into one or more host cells using, for example, chemically competent or electrocompetent cells or using known transfection reagents and techniques. In a preferred aspect, such manipulation involves RT-PCR, cDNA synthesis or cDNA library construction from RNA obtained from or contained by the solid support. Such manipulations according to the invention can be conducted after storage of the nucleic acids on the support or can be conducted directly without storage. The preferred medium or support is a matrix which protects against degradation of nucleic acids incorporated onto the matrix. Such a matrix may comprise an absorbent cellulose-based matrix or paper, or a micromesh of synthetic plastic material such as those described in U.S. Patent No. 5,496,562 and 5,976,572. Preferably, the matrix comprises a composition comprising a weak base, a chelating agent, an anionic surfactant or anionic detergent, and optionally uric acid or a urate salt, wherein said composition is absorbed on or incorporated into said matrix. FTA® paper (available from Life Technologies, Inc.) and derivatives, variants and modifications thereof are included among such supports. Also included are GenPrep™ and GenSpin™ available from Whatman and IsoCode™ available

from Schleicher and Schuell which may also be used according to the invention.

In the practice of the invention, any solid support may be used. Preferred such solid supports include, but are not limited to nitrocellulose, cellulose, diazocellulose, carboxymethylcellulose, hydrophilic polymers (e.g., polyester, polyamide, carbohydrate polymers), polytetra- fluro-ethylene, fiberglass, porous ceramics, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, and nylon.

According to the present invention, any nucleic acid molecules (e.g., RNA and DNA and particularly polyA+ RNA and mRNA) may be archived and later recovered and/or manipulated by a simple and efficient method in which a sample (e.g., cells, tissues, cellular materials, etc.) carrying the one or more nucleic acids are contacted with a solid medium (preferably FTA® paper or derivatives, variants or modifications thereof). In another aspect, purified nucleic acid molecules may be used, although in a preferred aspect, crude preparations (unpurified mRNA preparations or cell lysates) containing the one or more nucleic acid molecules may be contacted with the solid medium or support. Thus, any samples may provide the nucleic acid molecules to be contacted or bound to the support such as host cells, viruses, viral plaques, and/or crude preparations from biological materials (such as host cell or virus extracts, lysates, debris, hydrolysates, and the like). Such nucleic acid molecules obtained from or contained by the solid support or matrix may be used or manipulated in one or more standard molecular biology techniques, such as digestion, sequencing, amplification, synthesis and transformation/transfection reactions. Preferably, mRNA obtained from or contained by the solid support is used in RT-PCR or cDNA synthesis and particularly for cDNA library construction. In other preferred embodiments, the RNA obtained according to the invention may be used in Northern blots or attached to other solid supports, such as chips, for use in gene profiling applications. In a particularly preferred aspect, one or more host cells containing the nucleic acid molecules to be isolated, stored and/or manipulated can be contacted directly with the medium or support. According to the present invention, host cell cultures or colonies from plates may be used.

Preferred host cells for use in the invention include prokaryotic or eukaryotic host cells, particularly gram positive and gram negative bacteria, plant cells, animal cells (including human), insect cells and the like.

In the practice of the invention, nucleic acid molecules and in particular cDNA molecules or cDNA libraries are produced by mixing one or more nucleic acid templates obtained from or contained by a solid support of the invention (e.g., a mRNA molecule or a polyA+ RNA molecule) with one or more polypeptides having polymerase activity and/or reverse transcriptase activity under conditions favoring synthesis of one or more nucleic acid molecules complementary to all or a portion of the templates.

Preferred polypeptides (*e.g.*, enzymes) having reverse transcriptase and/or polymerase activity to be used in the present invention include, but are not limited to, Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase, Human Immunodeficiency Virus (HIV) reverse transcriptase, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, *Thermus thennophilus (Tth)* DNA polymerase, *Thermus aquaticus (Taq)* DNA polymerase, *Thermotoga neopolitana (Tne)* DNA polymerase, *Thermotogamaritima (Tma)* DNA polymerase, *Thermococcus litoralis (Tli, e.g.,* VENT® brand) DNA polymerase, *Pyrococcus furiosus (Pfu)* DNA polymerase, *Pyrococcus* species GB-D (e.g., DEEPVENT™ brand) DNA polymerase, *Pyrococcus woosii* (Pwo) DNA polymerase, *Bacillus sterothermophilus (Bst)* DNA polymerase, *Sulfolobus acidocaldarius (Sac)* DNA polymerase, *Thermoplasma acidophilum (Tac)* DNA polymerase, *Thermus favus (Tfl/Tub)* DNA polymerase, *Thermus ruber (Tru)* DNA polymerase, *Thermus brockianus (e.g.,* DYNAZYME® brand) DNA polymerase, *Methanobacterium thermoautotrophicuin (Mth)* DNA polymerase, and mutants, variants and derivatives thereof. Particularly preferred for use in the invention are the variants of these enzymes that are substantially reduced in RNase H activity. Preferred reverse transcriptases for

use in the invention include SUPERSCRIPT™, SUPERSCRIPT™ II and THERMOSCRIPT™ brands of reverse transcriptases available from the Life Technologies Division of Invitrogen Corporation (Rockville, MD), and other reverse transcriptases described in U.S. Patent 5,244,797 and WO 98/47912. By an enzyme "substantially reduced in RNase H activity" is meant that the enzyme has less than about 20%, more preferably less than about 15%, 10% or 5%, and most preferably less than about 2%, of the RNase H activity of a wildtype or "RNase H+" enzyme such as wildtype M-MLV or AMV reverse transcriptases. The RNase H activity of any enzyme may be determined by a variety of assays, such as those described, for example, in U.S. Patent No. 5,244,797, in Kotewicz, M.L., *et al., Nucl. Acids Res.* 16:265 (1988) and in Gerard, G.F., *et al., FOCUS* 14(5):91 (1992), the disclosures of all of which are fully incorporated herein by reference.

The invention is thus directed to methods for making one or more nucleic acid molecules, comprising mixing one or more nucleic acid templates (preferably one or more RNA templates and most preferably one or more messenger RNA templates) with one or more polypeptides having reverse transcriptase activity and incubating the mixture under conditions sufficient to make one or more first nucleic acid molecules complementary to all or a portion of the one or more nucleic acid templates. Such conditions preferably comprise the use of one or more primers (preferably oligo dT) and one or more nucleotides. In a preferred embodiment, the first nucleic acid molecule is a single-stranded cDNA. Nucleic acid templates suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule or population of nucleic acid molecules (preferably RNA and most preferably mRNA), particularly those derived from a cell or tissue. In a preferred aspect, a population of mRNA molecules (a number of different mRNA molecules, typically obtained from cells or tissue) are used to make a cDNA library, in accordance with the invention. Preferred cellular sources of nucleic acid templates include bacterial cells, fungal cells, plant cells and animal cells.

The invention also concerns methods for making one or more double-stranded nucleic acid molecules. Such methods comprise (a) mixing one or

more nucleic acid templates (preferably RNA or mRNA, and more preferably a population of mRNA templates) with one or more polypeptides having reverse transcriptase activity; (b) incubating the mixture under conditions sufficient to make one or more first nucleic acid molecules complementary to all or a portion of the one or more templates; and (c) incubating the first nucleic acid molecules under conditions sufficient to make one or more second nucleic acid molecules complementary to all or a portion of the first nucleic acid molecules, thereby forming one or more double-stranded nucleic acid molecules comprising the first and second nucleic acid molecules. Such methods may include the use of one or more DNA polymerases (and preferably one or more primers and nucleotides) as part of the process of making the one or more double-stranded nucleic acid molecules.

The invention also relates to methods for amplifying a nucleic acid molecule. Such amplification methods comprise mixing the double-stranded nucleic acid molecules produced as described above with one or more DNA polymerases and incubating the mixture under conditions sufficient to amplify the double-stranded nucleic acid molecule. In a first preferred embodiment, the invention concerns a method for amplifying one or more nucleic acid molecules, the method comprising (a) mixing one or more nucleic acid templates (preferably one or more RNA or mRNA templates and more preferably a population of mRNA templates) with one or more polypeptides having reverse transcriptase activity and with one or more DNA polymerases and (b) incubating the mixture under conditions sufficient to amplify nucleic acid molecules complementary to all or a portion of the one or more templates.

The invention is also directed to nucleic acid molecules (particularly single- or double-stranded cDNA molecules) or amplified nucleic acid molecules produced according to the above-described methods and to vectors (particularly expression vectors) comprising these nucleic acid molecules or amplified nucleic acid molecules.

The invention is further directed to compositions made or prepared while carrying out the methods of the invention. Such compositions may comprise the solid support of the invention, one or more mRNA molecules and/or one or more cDNA molecules produced from said mRNA molecules.

The invention is also directed to kits for use in the methods of the invention. Such kits can be used for making or amplifying nucleic acid molecules (single- or double-stranded) according to the invention. The kits of the invention comprise a carrier, such as a box or carton, having in close confinement therein one or more containers, such as vials, tubes, bottles and the like. Kits of the invention may comprise one or more of the reverse transcriptase enzymes (preferably one or more such enzymes that are reduced or substantially reduced in RNase H activity), one or more solid supports, one or more primers, one or more nucleotides and one or more reaction buffers. The kits of the invention may also comprise instructions for carrying out the methods of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of a Northern blot analysis of RNA stored and eluted from a solid support.

Figure 2 shows the results of an RT-PCR analysis of RNA stored and eluted from a solid support using samples derived from HeLa cells.

Figure 3 shows the results of an RT-PCR analysis of RNA stored and eluted from a solid support using samples derived from plant cells.

Figure 4 shows the results of an RT-PCR analysis of RNA from varying amounts of cells stored and eluted from a solid support using samples derived from HeLa cells.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and more consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Amplification. As used herein, "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a nucleic acid (*e.g.*, DNA) molecule or primer thereby forming a new nucleic acid molecule complementary to the nucleic acid template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of nucleic acid synthesis. Amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a nucleic acid molecule.

Polymerases (including DNA polymerases and RNA polymerases) useful in accordance with the present invention include, but are not limited to, *Thermus thermophilus (Tth)* DNA polymerase, *Thermus aquaticus (Taq)* DNA polymerase, *Thermotoga neopolitana (Tne)* DNA polymerase, *Thermotoga maritima (Tma)* DNA polymerase, *Thermococcus litoralis (Tli, e.g.,* VENT® brand) DNA polymerase, *Pyrococcus furiosus (Pfu)* DNA polymerase, Pyrococcus species GB-D (e.g., DEEPVENT™ brand) DNA polymerase, Pyrococcus woosii (Pwo) DNA polymerase, *Bacillus sterothermophilus (Bst)* DNA polymerase, *Bacillus caldophilus (Bca)* DNA polymerase, *Sulfolobus acidocaldarius (Sac)* DNA polymerase, *Thennoplasma acidophilian (Tac)* DNA polymerase, *Thennus flavus (Tfl/Tub)* DNA polymerase, *Thernius ruber (Tru)* DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicion (Mth)* DNA polymerase, mycobacterium DNA polymerase *(Mtb, Mlep),* and mutants, and variants and derivatives thereof. RNA polymerases such as T3, T5 and SP6 and mutants, variants and derivatives thereof may also be used in accordance with the invention.

Polymerases used in accordance with the invention may be any enzyme that can synthesize a nucleic acid molecule from a nucleic acid template, typically in the 5' to 3' direction. The nucleic acid polymerases used in the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include T7 DNA polymerase, T5 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III and the like. Preferred thermostable DNA polymerases that may be used in the methods of the invention include *Taq, Tne, Tma, Pfu, Tfl, Tth,* Stoffel fragment, *Tli* (e.g., VENT® brand) and *Pyrococcus* species GB-D DNA (e.g., DEEPVENT™ brand) polymerases, and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent 4,889,818; U.S. Patent 4,965,188; U.S. Patent 5,079,352; U.S. Patent 5,614,365; U.S. Patent 5,374,553; U.S. Patent 5,270,179; U.S. Patent 5,047,342; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene* 112:29-35 (1992); Lawyer, F.C., *et al., PCR Meth. Appl.* 2:275-287 (1993); Flaman, J.-M, *et al., Nucl. Acids Res.* 22(15):3259-3260 (1994)). For

amplification of long nucleic acid molecules (e.g., nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. *See U.S.* Patent No. 5,436,149; and U.S. Patent No. 5,512,462; Barnes, W.M., *Gene* 112:29-35 (1992), the disclosures of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in 3' exonuclease activity include, but are not limited to, *Taq, Tne(exo⁻), Tma(exo⁻), Pfu(exo⁻), Pwo(exo⁻)* and *Tth DNA* polymerases, and mutants, variants and derivatives thereof.

Host Cell. Any prokaryotic or eukaryotic cell. Such cell may be the recipient of a replicable expression vector or cloning vector. The terms "host" or "host cell" or "cell" may be used interchangeably herein. For examples of such hosts, *see* Maniatis *et al., Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Preferred prokaryotic hosts include, but are not limited to, bacteria of the genus *Escherichia (e.g.* E. coli), *Bacillus, Staphylococcus, Agrobacter (e.g. A. tumefaciens), Streptomyces, Pseudomonas, Salmonella, Serratia, Caryophanon,* etc. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest in the present invention include *E. coli* K12, DH10B, DH5a and HB101. Preferred eukaryotic hosts include, but are not limited to, fungi, fish cells, yeast cells, plant cells and animal cells. Particularly preferred animal cells are insect cells such as *Drosophila* cells, *Spodoptera* Sf9 and Sf21 cells and *Trichoplusa* High-Five cells; nematode cells such as C. *elegans* cells; and mammalian cells such as COS cells, CHO cells, VERO cells, 293 cells, PERC6 cells, BHK cells and human cells.

Vector. A vector is a nucleic acid molecule (preferably DNA) capable of replicating autonomously in a host cell. Such vectors may also be characterized by having a small number of endonuclease restriction sites at which such sequences may be cut without loss of an essential biological function and into which nucleic acid molecules may be spliced to bring about its replication and cloning. Examples include plasmids, autonomously replicating sequences (ARS), centromeres, cosmids and phagemids. Vectors can further provide primer sites, *e.g.*, for PCR, transcriptional and/or

translational initiation and/or regulation sites, recombinational signals, replicons, *etc.* The vector can further contain one or more selectable markers suitable for use in the identification of cells transformed or transfected with the vector, such as kanamycin, tetracycline, amplicillin, etc.

In accordance with the invention, any vector may be used. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be used in accordance with the invention. Such vectors may be obtained from, for example, Vector Laboratories Inc., Invitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OnGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. Such vectors may then for example be used for cloning or subcloning nucleic acid molecules of interest. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, transcription vectors, vectors for receiving large inserts and the like.

Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage 1 vectors, baculovirus vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors which have compatible replicons for use in combination in a single host (pACYC 184 and pBR322) and eukaryotic episomal replication vectors (pCDM8).

Storage. As used herein, "storage" refers to maintaining the support/nucleic acids for a period of time at a temperature or temperatures of interest. Preferably, storage is accomplished at about 20 to 30°C (preferably room temperature, e.g. 25°C), but may be at higher or lower temperatures depending on the need. Lower storage temperatures may range from about 0 to 20°C, -20 to 0°C, and -80 to - 20°C. Long term storage in accordance with the invention is greater than one year, preferably greater than 2 years, still more preferably greater than 3 years, still more preferably greater than 5 years, still more preferably greater than 10 years, and most preferably greater than 15 years.

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary sill in the applicable arts.

Production of cDNA Molecules

Sources of Nucleic Acid Molecules

In accordance with the invention, cDNA molecules (single-stranded or double-stranded) may be prepared from a variety of nucleic acid template molecules. Preferred nucleic acid molecules for use in the present invention include single-stranded or double-stranded DNA and RNA molecules, as well as double-stranded DNA:RNA hybrids. More preferred nucleic acid molecules include messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) molecules, although mRNA molecules are the preferred template according to the invention.

The nucleic acid molecules that are used to prepare cDNA molecules according to the methods of the present invention may be prepared synthetically according to standard organic chemical synthesis methods that will be familiar to one of ordinary skill. More preferably, the nucleic acid molecules may be obtained from natural sources, such as a variety of cells, tissues, organs or organisms. Cells that may be used as sources of nucleic acid molecules may be prokaryotic (bacterial cells, including but not limited to those of species of the genera *Escherichia, Bacillus, Serratia, Salmonella, Staphylococcus, Streptococcus, Clostridium, Chlamydia, Neisseria, Treponema, Mycoplasma, Borrelia, Legionella, Pseudomonas, Mycobacterium, Helicobacter, Erwinia, Agrobacterium, Rhizobium, Xanthomonas* and *Streptomyces)* or eukaryotic (including fungi (especially yeasts), plants, protozoans and other parasites, and animals including insects including but not limited to *Drosophila* spp. cells, *Spodoptera* Sf9 and Sf21 cells and *Trichoplusa* High-Five cells; nematodes (particularly *Caenorhabditis elegans* cells), and mammals such as COS cells, CHO cells, VERO cells, 293 cells, PERC6 cells, BHK cells, and other mouse and human cells. In some preferred embodiments, the nucleic acids stored according to the present invention may be derived from viruses.

Mammalian somatic cells that may be used as sources of nucleic acids include blood cells (reticulocytes and leukocytes), endothelial cells, epithelial cells, neuronal cells (from the central or peripheral nervous systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other stromal cells (e.g., macrophages, dendritic cells, Schwann cells). Mammalian germ cells (spermatocytes and oocytes) may also be used as sources of nucleic acids for use in the invention, as may the progenitors, precursors and stem cells that give rise to the above somatic and germ cells. Also suitable for use as nucleic acid sources are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus.

Any of the above prokaryotic or eukaryotic cells, tissues and organs may be normal, diseased, transformed, established, progenitors, precursors, fetal or embryonic. Diseased cells may, for example, include those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS, HIV, HTLV, herpes, hepatitis and the like) or parasites), in genetic or biochemical pathologies (*e.g.*, cystic fibrosis, hemophilia, Alzheimer's disease, muscular dystrophy or multiple sclerosis) or in cancerous processes. Transformed or established animal cell lines may include, for example, COS cells, CHO cells, VERO cells, BHK cells, HeLa cells, HepG2 cells, K562 cells, 293 cells, L929 cells, F9 cells, and the like. Other cells, cell lines, tissues, organs and organisms suitable as sources of nucleic acids for use in the present invention will be apparent to one of ordinary skill in the art.

Once the starting cells, tissues, organs or other samples are obtained, nucleic acid molecules (such as mRNA) may optionally be isolated therefrom by methods that are well-known in the art *(See, e.g.,* Maniatis, T., *et al., Cell* 15:687-701 (1978); Okayama, H., and Berg, P., *Mol. Cell. Biol.* 2:161-170 (1982); Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983)). The nucleic acid molecules thus isolated may then be contacted directly with the solid

supports of the invention. Alternatively, cells, tissues, etc., may be contacted directly with the support.

In the practice of the invention, cDNA molecules or cDNA libraries are produced by mixing one or more nucleic acid molecules obtained as described above, which is preferably one or more mRNA molecules such as a population of mRNA molecules, with one or more polypeptides having reverse transcriptase activity under conditions favoring the reverse transcription of the nucleic acid molecule by the action of the enzymes to form one or more cDNA molecules (single-stranded or double-stranded). Thus, the method of the invention comprises (a) mixing one or more nucleic acid templates (preferably one or more RNA or mRNA templates, such as a population of mRNA molecules) with one or more reverse transcriptases and (b) incubating the mixture under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the one or more templates. Such methods may include the use of one or more DNA polymerases. The invention may be used in conjunction with methods of cDNA synthesis such as those described in the Examples below, or others that are well-known in the art *(see, e.g.,* Gubler, U., and Hoffman, B.J., *Gene* 25:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol.152:316*-325 (1987); Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989); and WO 98/51699), to produce cDNA molecules or libraries. In a preferred embodiment, the cDNA may be produced using the methods detailed in United States patent application serial number 09/076,115 and/or United States provisional application serial number 60/122,395 filed March 2, 1999.

In pertinent part, United States patent application serial number 09/076,115 reads as follows:

" SUMMARY OF THE INVENTION

The present invention is directed to methods useful for the production and isolation of nucleic acid molecules (single- and double-stranded) from

small amounts of input nucleic acid molecules. More particularly, the invention provides methods for the production of a cDNA molecule (single- or double-stranded) from an RNA template (*e.g.*, single-stranded mRNA or polyA+ RNA) by using ligand-coupled primer-adapter molecules. Such primer-adapter molecules may also be used in accordance with the invention to isolate mRNA or polyA+ RNA molecules from an RNA-containing sample.

Specifically, the invention is directed to a method for producing a nucleic acid molecule comprising mixing a nucleic acid template, preferably a mRNA or a polyA+ RNA molecule, with a polypeptide having polymerase and/or reverse transcriptase activity and a primer-adapter nucleic acid molecule, wherein the primer-adapter nucleic acid molecule comprises one or more ligand molecules and one or more cleavage sites (preferably a restriction endonuclease cleavage site or an endonuclease cleavage site). This primer-adapter may be designed to hybridize to any portion of the template. Upon incubation under appropriate conditions, a first nucleic acid molecule (*e.g.*, a single-stranded cDNA) complementary to all or a portion of the template is made. This first nucleic acid molecule contains the primer-adapter (preferably at or near its termini) which facilitates isolation of the first nucleic acid molecule and/or any nucleic acid molecule hybridized to the first nucleic acid molecule. Thus, if the first nucleic acid molecule (*e.g.*, single-stranded cDNA) serves as a template to make a second nucleic acid molecule (*e.g.*, forming a double-stranded molecule such as a double-stranded cDNA), the double-stranded molecule can be isolated using the primer-adapter contained in the molecule. Likewise, the template-first nucleic acid hybrid formed during synthesis of the first nucleic acid molecule can be isolated. If desired, the primer-adapter may be included at any step or at multiple steps during nucleic acid synthesis. For example, primer-adapter molecules may be added during the first, second, third, fourth, etc., synthesis step (the first synthesis step making a nucleic acid molecule complementary to all or a portion of the template) or can be added in multiple or all such synthesis steps. Multiple synthesis with primer-adapter molecules may result in a synthesized nucleic acid molecule having more than one primer-adapter.

To isolate mRNA or polyA+ RNA from RNA-containing samples, one or more mRNA- or polyA+ RNA-specific primer-adapters is used. Such a primer-adapter is hybridized to the mRNA and/or polyA+ RNA to form a primer-adapter/polyA+ RNA hybrid. The primer-adapter can then facilitate isolation of the mRNA and/or polyA+ RNA from a sample. In this aspect of the invention, since the primer-adapter is hybridized to the molecule of interest and can be removed by denaturation, cleavage sites in the primer-adapter are not needed.

The primer-adapter molecules of the invention may also be used to isolate specific nucleic acid sequences. By using one or more target-specific primer-adapters capable of hybridizing to one or more sequences of interest, the invention allows selection and isolation of specific nucleic acid molecules (*e.g.*, genes or portions thereof) from a population of nucleic acid molecules. In accordance with the invention, the use of two or more such target-specific primer-adapters (each directed to a different sequence) allows selection of more than one different sequence of interest. Alternatively, two or more target-specific primer-adapters directed to different portions of a sequence of interest facilitates selection of such sequences by reducing background contamination. Because, in this aspect of the invention, the target-specific primer-adapter hybridizes to the desired molecule and can be removed by denaturation, cleavage sites in the target-specific primer-adapter are not needed.

In accordance with the invention, the primer-adapter molecules facilitate isolation of molecules comprising such primer-adapters by relying on the ligand portion of the primer-adapter. After the primer-adapter is bound (hybridized or incorporated during synthesis) to the nucleic acid molecule, the ligand portion of the primer-adapter allows selective isolation of the molecule containing the primer-adapter. Such isolation may be accomplished by ligand-hapten interactions, where the hapten is bound to, for example, a solid support. Once bound to the solid support, the molecules of interest (primer-adapter containing nucleic acid molecules) can be separated from contaminating nucleic acids and proteins by washing the support matrix with a solution, preferably a buffer or water. Cleavage of one or more of the cleavage sites

within the primer-adapter then allows for removal of the nucleic acid molecule of interest from the solid support, leaving the ligand bound to the hapten of the solid support. Alternatively, where the primer-adapter is hybridized to the nucleic acid molecule of interest, isolation can be accomplished by denaturation of the primer-adapter from the desired molecules and/or by cleavage of the cleavage sites within the primer-adapter molecule.

Preferred solid supports for use in the invention include, but are not limited to, nitrocellulose, diazocellulose, glass, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, nylon, latex beads, magnetic beads, paramagnetic beads, superparamagnetic beads or microtitre plates and most preferably a magnetic bead, a paramagnetic bead or a superparamagnetic bead, that comprises one or more hapten molecules specifically recognizing and binding to the ligand molecule.

Particularly preferred hapten molecules according to this aspect of the invention include without limitation: (i) avidin and streptavidin; (ii) protein A, protein G, a cell-surface Fc receptor or an antibody- specific antigen; (iii) an enzyme-specific substrate; (iv) polymyxin B or endotoxin-neutralizing protein (ENP); (v) Fe^{+++}; (vi) a transferrin receptor; (vii) an insulin receptor; (viii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (ix) CD4; (x) spectrin or fodrin; (xi) ICAM-1 or ICAM-2; (xii) C3bi, fibrinogen or Factor X; (xiii) ankyrin; (xiv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xvi) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xvii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xviii) vitronectin; (xix) fibronectin; (xx) collagen; (xxi) laminin; (xxii) glycophorin; (xxiii) Mac-1; (xxiv) LFA-1; (xxv) β-actin; (xxvi) gp120; (xxvii) cytokines (growth factors, interleukins or colony-stimulating factors); (xxviii) insulin; (xxix) ferrotransferrin; (xxx) apotransferrin; (xxxi) lipopolysaccharide; (xxxii) an enzyme; (xxxiii) an antibody; and (xxxiv) biotin.

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The invention thus relates to a method for making a nucleic acid molecule comprising

(a) mixing a polypeptide having polymerase and/or reverse transcriptase activity with a nucleic acid template and a primer-adapter of the invention; and

(b) incubating the mixture under conditions sufficient to make a first nucleic acid molecule which comprises the primer-adapter (preferably at or near its 5' or 3' termini) and which is complementary to all or a portion of the template. If a DNA polymerase is used in accordance with the invention, the primer-adapter may be located at or near the 3' terminus, while if a reverse transcriptase is used the primer-adapter may be located at or near the 5' terminus of the synthesized nucleic acid molecule. In accordance with the invention, the first nucleic acid molecule may be used as a template to make a second nucleic acid molecule complementary to all or a portion of the first nucleic acid molecule. If a primer-adapter is used in this synthesis, a double-stranded nucleic acid molecule is produced which comprises a primer-adapter at or near each terminus, although on different strands of the molecule. However, the primer-adapter may be omitted from this second synthesis thereby providing for a double-stranded nucleic acid molecule having a primer-adapter at one terminus.

If desired, the primer-adapters of the invention may be used in methods for amplifying a nucleic acid molecule. Such methods comprise

(a) contacting a polypeptide having polymerase and/or reverse transcriptase activity with a nucleic acid template and two or more primer-adapters; and

(b) incubating the mixture under conditions sufficient to amplify a nucleic acid molecule complementary to all or a portion of the template.

Such amplification methods may specifically comprise

(a) contacting a double-stranded nucleic acid molecule to be amplified with a polypeptide having polymerase and/or reverse transcriptase activity, a first primer-adapter complementary to a portion of the first strand of the double-stranded molecule and a second primer-adapter complementary to a portion of the second strand of the double-stranded molecule;

(b) incubating the mixture under conditions sufficient to make a third strand nucleic acid molecule comprising the first primer-adapter and

which is complementary to all or a portion of the first strand, and a fourth strand nucleic acid molecule comprising the second primer-adapter and which is complementary to all or a portion of the second strand;

(c) denaturing the second and fourth, and the first and third, strands to form single-stranded nucleic acid molecules; and

(d) repeating steps (a)-(c) one or more times.

In this aspect of the invention, the first primer-adapter or the second primer-adapter may be replaced with any oligonucleotide primer to prime synthesis of a nucleic acid molecule.

In a preferred aspect of the invention, RNA (*e.g.*, mRNA or polyA+ RNA) is used as a template for DNA synthesis. This preferred method comprises mixing the RNA template with one or more polypeptides having reverse transcriptase activity and a primer and incubating the mixture under conditions sufficient to make a DNA (*e.g.*, a cDNA) molecule complementary to all or a portion of the RNA template. The synthesized DNA molecule may then be used as a template for additional DNA synthesis or DNA amplification. In accordance with this aspect of the invention, a cDNA library may be produced when using a population of RNA molecules (for example, RNA isolated from a cell or tissue).

For isolating mRNA or polyA+ RNA in accordance with the invention, the method may specifically comprise:

(a) obtaining a sample containing (or thought to contain) mRNA and/or polyA+ RNA;

(b) contacting the sample with one or more primer-adapters capable of selectively binding to mRNA and/or polyA+ RNA; and

(c) isolating the mRNA and/or polyA+ RNA from the sample.

For isolating specific or desired nucleic acid molecules, the invention may specifically comprise:

(a) obtaining a sample containing (or thought to contain) one or more desired nucleic acid molecules;

(b) contacting the sample with one or more primer-adapters capable of selectively binding to one or more of the desired nucleic acid molecules; and

(c) isolating the desired nucleic acid molecules from the sample.

In a preferred aspect, the sample containing the desired molecules is a population of double-stranded or single-stranded cDNA molecules. Thus, the invention relates to a method of isolating one or more desired nucleic acid molecules comprising:

(a) obtaining a sample containing a population of cDNA molecules which contain (or are thought to contain) one or more desired cDNA molecules;

(b) contacting the sample with one or more target-specific primer-adapters capable of specifically binding to one or more of the desired cDNA molecules; and

(c) isolating the desired cDNA molecules from the sample.

In accordance with the invention, the target-specific primer-adapters may be used in selection of a specific cDNA molecule after the cDNA molecule is synthesized from the RNA template (binding to the RNA/cDNA double-stranded molecule or binding to the single-stranded cDNA molecule after removing the RNA strand). Alternatively, the target-specific primer-adapters may be used to bind the double-stranded cDNA molecule. Such target-specific primer-adapters may also be used in accordance with the invention to select one or more desired molecules from a population of amplified nucleic acid molecules.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention is particularly suited for the rapid production and isolation of cDNA libraries from small amounts of poly A+ RNA or mRNA in a high-throughout manner. In a preferred aspect of the invention, a population of single-stranded poly A+ RNA or mRNA is hybridized in solution with a ligand-coupled primer adapter (non-specific or gene-specific). As used herein, the term "primer-adapter" refers to a nucleic acid molecule which is capable of specifically binding (*e.g.*, hybridizing) to a template nucleic acid molecule (*e.g.*, a mRNA or polyA+ RNA molecule). In a particularly preferred

embodiment of the invention, the primer-adapter allows priming of the transcription, reverse transcription, polymerization or elongation of a nucleic acid molecule complementary to all or a portion of the template nucleic acid molecule.

According to the invention, the first and second strand cDNA reactions are preferably performed in one tube, introducing the ligand at or near the 3' end of the double-stranded cDNA produced. The ligand-coupled cDNA may then be isolated by binding to a solid support coupled with a hapten to which the cDNA will bind through ligand-hapten interactions, thereby allowing the concentration of the cDNA and exchange of the buffer without organic extraction and precipitation. Subsequently, the bound cDNA is released from the solid phase support by restriction enzyme digestion. This asymmetric cDNA is then cloned directionally into a vector that contains the appropriate termini (one terminus matches the restriction site used to release the cDNA and the other terminus is blunt ended). Subsequent or prior to cloning into a vector, specific cDNA sequences (*e.g.*, genes or gene fragments) may be selectively isolated using target-specific primer-adapters of the invention. In addition to the elimination of multiple time-consuming extractions and precipitations, the methods of the invention eliminate the need for DNA adapters and cDNA fractionation (normally a necessary step to remove excess unligated adapters). The invention thus facilitates rapid production and isolation of larger amounts of cDNA and the construction of cDNA libraries from nanogram amounts of poly A+ RNA or mRNA without the need for PCR amplification. The invention also provides a simple selection technique which allows isolation of desired genes or gene fragments from the constructed cDNA library.

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As discussed, the invention provides an improvement in isolating mRNA and/or polyA+ RNA from samples. The use of the primer-adapters of the invention, which specifically recognize and bind polyA+ RNA or mRNA, allows for such selection. Preferably, the primer-adapter recognizes and

hybridizes to the polyA tail of the mRNA or polyA+ RNA. Such primer-adapters may include an primer-adapters comprising oligo(dT). Once bound, use of the ligand portion of the primer-adapter allows isolation of the desired RNA molecule. The polyA+ RNA or mRNA molecules thus isolated may then be used to prepare cDNA molecules and cDNA libraries using the methods of the present invention.

Synthesis of Nucleic Acid Molecules

In the practice of the invention, nucleic acid molecules and in particular cDNA molecules or cDNA libraries comprising one or more ligand molecules are produced by mixing a nucleic acid template obtained as described above, which is preferably a mRNA molecule or a polyA+ RNA molecule, with one or more polypeptides having polymerase activity and/or reverse transcriptase activity and with a one or more primer-adapters of the invention. Under conditions favoring the reverse transcription and/or polymerization of the input nucleic acid molecule, synthesis of a nucleic acid molecule complementary to all or a portion of the template is accomplished. Preferred polypeptides (*e.g.*, enzymes) having reverse transcriptase and/or polymerase activity to be used in the present invention include, but are not limited to, Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase, Human Immunodeficiency Virus (HIV) reverse transcriptase, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, *Thermus thermophilus* (*Tth*) DNA polymerase, *Thermus aquaticus* (*Taq*) DNA polymerase, *Thermotoga neopolitana* (*Tne*) DNA polymerase, *Thermotoga maritima* (*Tma*) DNA polymerase, *Thermococcus litoralis* (*Tli* or VENT™) DNA polymerase, *Pyrococcus furiosus* (*Pfu* or DEEPVENT™) DNA polymerase, *Pyrococcus woosii* (*Pwo*) DNA polymerase, *Bacillus sterothermophilus* (*Bst*) DNA polymerase, *Sulfolobus acidocaldarius* (*Sac*) DNA polymerase, *Thermoplasma acidophilum* (*Tac*)

DNA polymerase, *Thermus flavus* (*Tfl/Tub*) DNA polymerase, *Thermus ruber* (*Tru*) DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum* (*Mth*) DNA polymerase, and mutants, variants and derivatives thereof. Particularly preferred for use in the invention are the variants of these enzymes that are substantially reduced in RNase H activity. By an enzyme "substantially reduced in RNase H activity" is meant that the enzyme has less than about 20%, more preferably less than about 15%, 10% or 5%, and most preferably less than about 2%, of the RNase H activity of a wildtype or "RNase H^+" enzyme such as wildtype M-MLV or AMV reverse transcriptases. The RNase H activity of any enzyme may be determined by a variety of assays, such as those described, for example, in U.S. Patent No. 5,244,797, in Kotewicz, M.L., *et al.*, *Nucl. Acids Res. 16*:265 (1988) and in Gerard, G.F., *et al.*, *FOCUS 14(5)*:91 (1992), the disclosures of all of which are fully incorporated herein by reference.

Any ligand to which a hapten molecule will bind may be used to form the ligand-coupled primer-adapter molecule used in the present methods. Suitable ligands for this purpose include, but are not limited to: (i) biotin; (ii) an antibody; (iii) an enzyme; (iv) lipopolysaccharide; (v) apotransferrin; (vi) ferrotransferrin; (vii) insulin; (viii) cytokines (growth factors, interleukins or colony-stimulating factors); (ix) gp120; (x) β-actin; (xi) LFA-1; (xii) Mac-1; (xiii) glycophorin; (xiv) laminin; (xv) collagen; (xvi) fibronectin; (xvii) vitronectin; (xviii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xix) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xx) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xxi) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xxii) ankyrin; (xxiii) C3bi, fibrinogen or Factor X; (xxiv) ICAM-1 or ICAM-2; (xxv) spectrin or fodrin; (xxvi) CD4; (xxvii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (xxviii) an insulin receptor; (xxix) a transferrin receptor; (xxx) Fe^{+++}; (xxxi) polymyxin B or endotoxin-neutralizing protein (ENP); (xxxii) an enzyme-specific substrate; (xxxiii) protein A, protein G, a cell-surface Fc receptor or an antibody-specific antigen; and (xxxiv) avidin and streptavidin. Most preferred for use in the methods of the invention is biotin. The ligand-coupled primer-adapter nucleic acid molecules, in which

one or more ligand molecules are attached (preferably covalently) to one or more nucleotides of the primer-adapter molecule * * * may be produced using conventional organic synthesis methods that are familiar to one of ordinary skill in the art. For example, the oligonucleotide may be biotinylated at the 5' terminus by first producing 5' amino (NH_2) groups followed by Cab-NHS ester addition (Langer, P.R., *et al.*, *Proc. Natl. Acad. Sci. USA 78*:6633 (1981)). In a particularly preferred aspect of the invention, a primer-adapter molecule comprising one or more, two or more, three or more or four or more ligand molecules, most preferably biotin molecules, is prepared.

In addition to the ligand molecules, the primer-adapter molecule also preferably comprises one or more endonuclease cleavage sites, preferably restriction endonuclease cleavage sites. These sites facilitate the release of the newly synthesized nucleic acid molecule comprising the primer-adapter from the hapten-coupled solid support. Examples of endonucleases which can be used in accordance with the invention include, but are not limited to, GeneII. Examples of restriction endonucleases which can be used in accordance with the invention include, but are not limited to, *Alu*I, *Eco*47 III, *Eco*RV, *Fsp*I, *Hpa*I, *Msc*I, *Nru*I, *Pvu*II, *Rsa*I, *Sca*I, *Sma*I, *Ssp*I, *Stu*I, *Tha*I, *Ava*I, *Bam*HI, *Ban*II, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Hpa*II, *Kpn*I, *Mse*I, *Nco*I, *Nde*I, *Not*I, *Pst*I, *Pvu*I, *Sac*I/*Sst*I, *Sal*I, *Xba*I, *Xho*I and I-*Ceu*I.

The restriction endonuclease sites engineered into the primer-adapter molecule are preferably chosen to result in either blunt ends or sticky ends. Examples of blunt-end restriction enzymes, the recognition sites for which may be engineered into the primer-adapter molecules of the invention, include without limitation *Alu*I, *Eco*47 III, *Eco*RV, *Fsp*I, *Hpa*I, *Msc*I, *Nru*I, *Pvu*II, *Rsa*I, *Sca*I, *Sma*I, *Ssp*I, *Stu*I and *Tha*I.

Examples of sticky-end restriction enzymes, the recognition sites for which may be engineered into the primer-adapter molecules of the invention, include without limitation *Ava*I, *Bam*HI, *Ban*II, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Hpa*II, *Kpn*I, *Mse*I, *Nco*I, *Nde*I, *Not*I, *Pst*I, *Pvu*I, *Sac*I/*Sst*I, *Sal*I, *Xba*, *Xho*I and I-*Ceu*I.

In a particularly preferred aspect of the invention, the primer-adapter molecule is engineered to contain a site recognized by rare cutting restriction

endonucleases, for example, those recognizing 8 or more bases (*e.g.*, a 8-basepair cutter, etc.). Such restriction sites may include a *Not*I restriction site, a I-*Ceu*I restriction site, a PI-*Psp*I restriction site, an I-*Ppo*I restriction site, a PI-*Tli*I restriction site and a PI-*Fce*I restriction site. The above-mentioned restriction enzymes, and others that may be equivalently used in the methods of the present invention, are available commercially, for example from Life Technologies, Inc. (Rockville, MD). See also Roberts, R.J., *Nucl. Acids Res. 17*(Suppl.):r347-r387 (1989), for other examples of restriction enzymes and their cleavage sites.

Once the ligand-coupled primer-adapter molecule has been obtained, it is used to produce nucleic acid molecules from the input nucleic acid using any of a number of well-known techniques. Such synthetic techniques involve hybridization of the primer-adapter to the nucleic acid template and extending the primer-adapter to make a nucleic acid molecule complementary to all or a portion of the template. Such synthesis is accomplished in the presence of nucleotides (*e.g.*, deoxyribonucleoside triphosphates (dNTPs), dideoxyribonucleoside triphosphates (ddNTPs) or derivatives thereof) and one or more polypeptides having polymerase and/or reverse transcriptase activity. The primer-adapters of the invention may be used in any nucleic acid synthesis reaction including cDNA synthesis, nucleic acid amplification and nucleic acid sequencing, using well-known techniques. For synthesis of cDNA, the primer-adapter molecules of the invention may be used in conjunction with methods of cDNA synthesis such as those described in Example 1 below, or others that are well-known in the art (*see, e.g.*, Gubler, U., and Hoffman, B.J., *Gene 25*:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol. 152*:316-325 (1987); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1987)), to produce cDNA molecules or libraries.

Alternatively, the primer adapter molecules of the invention may be used in single-tube synthesis of cDNA molecules according to the invention. In this approach, the input nucleic acid molecule (preferably a mRNA or polyA+ RNA molecule) is hybridized in solution with the primer-adapter

molecule of the invention, and the hybridized complex is contacted with a polypeptide (*e.g.*, an enzyme) having reverse transcriptase activity (which is preferably any of those described above) in the presence of dNTPs and cofactors needed for cDNA synthesis. Following first strand synthesis, the second cDNA strand may then be synthesized in the same reaction vessel by a modified Gubler-Hoffman reaction (D'Alessio, J.M., *et al.*, *Focus 9*:1 (1987)). Other techniques of cDNA synthesis in which the methods of the invention may be advantageously used will be readily apparent to one of ordinary skill in the art.

Isolation of Nucleic Acid Molecules

According to the present methods, single-stranded or double-stranded nucleic acid molecules (*e.g.*, cDNA molecules or cDNA libraries) comprising one or more primer-adapters will be produced. Such nucleic acid molecules or libraries may then be rapidly isolated from solution by binding the nucleic acid molecules to a solid support comprising one or more hapten molecules that will bind the ligands.

In the practice of the invention, any solid support to which a ligand-specific hapten molecule can be bound may be used. Preferred such solid phase supports include, but are not limited to, nitrocellulose, diazocellulose, glass, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, nylon, beads and microtitre plates. Preferred are beads made of glass, latex or a magnetic material, and particularly preferred are magnetic, paramagnetic or superparamagnetic beads. Linkage of the hapten molecule to the solid support can be accomplished by any method of hapten coupling such as covalent, hydrophobic or ionic coupling (including coating) that will be familiar to one of ordinary skill in the art.

According to the invention, any hapten molecule having the capability of binding the ligand molecule that is coupled to the primer-adapter molecule (and that therefore is contained in the nucleic acid molecules produced by the present methods) may be used. Particularly preferred hapten molecules for use in the invention (which correspond in order to the ligand molecules listed above) include without limitation: (i) avidin and streptavidin; (ii) protein A,

protein G, a cell-surface Fc receptor or an antibody- specific antigen; (iii) an enzyme-specific substrate; (iv) polymyxin B or endotoxin-neutralizing protein (ENP); (v) Fe^{+++}; (vi) a transferrin receptor; (vii) an insulin receptor; (viii) a cytokine (*e.g.*, growth factor, interleukin or colony-stimulating factor) receptor; (ix) CD4; (x) spectrin or fodrin; (xi) ICAM-1 or ICAM-2; (xii) C3bi, fibrinogen or Factor X; (xiii) ankyrin; (xiv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_5$; (xv) integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$; (xvi) integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$; (xvii) integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$; (xviii) vitronectin; (xix) fibronectin; (xx) collagen; (xxi) laminin; (xxii) glycophorin; (xxiii) Mac-1; (xxiv) LFA-1; (xxv) β-actin; (xxvi) gp120; (xxvii) cytokines (growth factors, interleukins or colony-stimulating factors); (xxviii) insulin; (xxix) ferrotransferrin; (xxx) apotransferrin; (xxxi) lipopolysaccharide; (xxxii) an enzyme; (xxxiii) an antibody; and (xxxiv) biotin.

For example, in a preferred aspect of the invention where the primer-adapter molecule and the newly synthesized nucleic acid molecules comprise biotin, a biotin-binding hapten such as avidin or streptavidin may be linked to the solid support. In a particularly preferred such aspect, the solid support used is avidin- or streptavidin-coupled magnetic, paramagnetic or superparamagnetic beads which are commercially available, for example, from Dynal A.S. (Oslo, Norway) or from Sigma (St. Louis, Missouri). Of course, the choice of hapten will depend upon the choice of ligand used in the production of the primer-adapter molecule; appropriate haptens for use in the methods of the invention will thus be familiar to one of ordinary skill in the art.

To isolate the nucleic acid molecules produced by the methods of the invention, the solution comprising the nucleic acid molecules which comprise the primer-adapters of the invention is contacted with the hapten-coupled solid support under conditions favoring binding of the ligand by the hapten. Typically, these conditions include incubation in a buffered salt solution, preferably a TRIS-, phosphate-, HEPES- or carbonate-buffered sodium chloride solution, more preferably a TRIS-buffered sodium chloride solution, still more preferably a solution comprising about 10-100 mM TRIS-HCl and

about 300-2000 mM NaCl, and most preferably a solution comprising about 10 mM TRIS-HCl and about 1 M NaCl, at a pH of about 6-9, more preferably a pH of about 7-8, still more preferably a pH of about 7.2-7.6, and most preferably a pH of about 7.5. Incubation is preferably conducted at 0°C to about 25°C, and most preferably at about 25°C, for about 30-120 minutes, preferably about 45-90 minutes, and most preferably about 60 minutes, to allow the binding of the ligand-coupled nucleic acid molecules to the hapten-coupled solid support.

Once the nucleic acid molecules have been bound to the solid phase support, unwanted or contaminant materials (such as buffers and enzymes from first and second strand synthesis reactions, untranscribed input RNA molecules, etc.) may be eliminated by simply removing them in the supernatants. For example, in a preferred aspect in which biotinylated cDNA molecules are bound to a avidin- or streptavidin-coupled solid phase, the contaminants may be removed by gently aspirating and discarding the supernatants. In a particularly preferred such aspect in which avidin- or streptavidin-coupled magnetic, paramagnetic or superparamagnetic beads are used as the solid support, the nucleic acid (*e.g.*, cDNA)-containing beads are segregated from the supernatants using a magnet (such as a Magna-Sep Magnetic Particle Separator; Life Technologies, Inc.) and the supernatants are withdrawn using a pipette. Prior to their release from the solid support, the immobilized nucleic acid molecules are preferably washed one or more times, for example with one of the buffered salt solutions described above, to more fully remove unwanted materials.

Once the contaminants have been fully removed, the nucleic acid (*e.g.*, cDNA) molecules may be released from the solid support by contacting the support with an endonuclease, which may be a restriction endonuclease, that specifically recognizes the sequence engineered into the primer-adapter molecule as described above, under conditions favoring the cleavage of the recognition sequence. In a particularly preferred such aspect of the invention in which a *Not*I and/or I-*Ceu*I recognition sequence is engineered into the primer-adapter molecule (and is thus contained in the newly synthesized nucleic acid (*e.g.*, cDNA) molecules), the solid support is contacted with a

solution comprising *Not*I and/or I-*Ceu*I. Of course, the choice of restriction enzyme used to release the nucleic acid molecules from the solid support will depend upon the specific recognition site engineered into the primer-adapter molecule and the possibility of that recognition site being present in the nucleic acid molecules. Preferred conditions for release of the nucleic acid molecules (*e.g.*, cDNA or cDNA libraries) from the solid support include incubation at about 20°C to about 40°C, preferably at about 25°C to about 39°C, more preferably about 30°C to about 37°C, and most preferably about 37°C, for about 30-180 minutes, preferably about 60-150 minutes, and most preferably about 120 minutes. Following their release from the solid support, the nucleic acid molecules (*e.g.*, cDNA molecules or cDNA libraries) may be processed and further purified in accordance with the invention, or by techniques that are well-known in the literature (*see*, *e.g.*, Gubler, U., and Hoffman, B.J., *Gene 25*:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol. 152*:316-325 (1987); Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1987)), and others that will be familiar to one of ordinary skill in the art.

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Uses

The present invention can be used in a variety of applications requiring rapid production and isolation of nucleic acid molecules. The invention is particularly suited for isolation of mRNA or polyA+ RNA molecules, for isolation of desired nucleic acid molecules from a population of nucleic acid molecules, and for production of nucleic acid molecules (particularly full-length cDNA molecules from small amounts of mRNA).

The invention is also directed to methods for the amplification of a nucleic acid molecule, and to nucleic acid molecules amplified by to these methods. According to this aspect of the invention, a nucleic acid molecule may be amplified (*i.e.*, additional copies of the nucleic acid molecule prepared) by amplifying the nucleic acid molecule (*e.g.*, a cDNA molecules)

of the invention according to any amplification method that is known in the art. Particularly preferred amplification methods according to this aspect of the the invention include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). Most preferred are those methods comprising one or more PCR amplifications.

The invention is also directed to methods that may be used to prepare recombinant vectors which comprise the nucleic acid molecules or amplified nucleic acid molecules of the present invention, to host cells which comprise these recombinant vectors, to methods for the production of a recombinant polypeptide using these vectors and host cells, and to recombinant polypeptides produced using these methods.

Recombinant vectors may be produced according to this aspect of the invention by inserting, using methods that are well-known in the art, one or more of the nucleic acid molecules or amplified nucleic acid molecules prepared according to the present methods into a vector * * * * The vector used in this aspect of the invention may be, for example, a phage or a plasmid, and is preferably a plasmid. Preferred are vectors comprising *cis*-acting control regions to the nucleic acid encoding the polypeptide of interest. Appropriate *trans*-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors are expression vectors that provide for specific expression of the cDNA molecule or nucleic acid molecule of the invention, which vectors may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

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In other applications, the methods of the invention may be used to generate a gene-specific cDNA library from a complex population of poly A+

RNA. The methods of the invention, in combination with polymorphism analysis methods such as AFLP, also facilitate rapid and direct identification of transcriptional differences between two different DNA populations. Additionally, the primer-adapter used in the invention can be designed to contain a regulatory sequence, such as a promoter, enhancer or other regulatory region. In one such aspect, a promoter for T7 or SP6 RNA polymerase may be engineered into the primer-adapter, thereby enabling the production of additional copies of the original mRNA for use in amplification or subtraction. Furthermore, the methods of the invention can be used to isolate poly A+ RNA from total RNA, such as from cells, tissues, organs or organisms, or to generate a cDNA library directly from total RNA. In the latter application, the invention is particularly useful when the mRNA of interest represents only a minute fraction of the total RNA; by the invention, this low-level mRNA may be rapidly and efficiently isolated from the background of total RNA and may then be rapidly and efficiently reverse transcribed into single-stranded or double-stranded cDNA molecules for a variety of purposes such as cloning and/or amplification.

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Example 1: ***Production and Isolation of cDNA Molecules***

First and second strand cDNA synthesis reactions were conducted as described in the instruction manual for the SUPERSCRIPT Plasmid System (Life Technologies, Inc., Rockville, Maryland), except that 50-5000 ng of mRNA was used as starting material to produce a library of $>10^6$ clones. The primer-adapter used in cDNA synthesis contained four biotin (B) residues: B-GACT (-B) AGT (-B)T(-B)CTAGATCGCGAGCGGCCGCCC(T_{15}) [(SEQ ID NO:14)].

Briefly, 1 μg of the biotinylated primer-adapter was used to prime first strand synthesis for 60 minutes, in a solution containing 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM each of dATP, dCTP, dGTP and dTTP, 50 μM/ml Bio-p-A and 10,000 to 50,000 units/ml SuperScript II reverse transcriptase (Life Technologies, Inc.). Second strand

synthesis was performed for two hours at 16°C using methods described previously (Okayama, H., and Berg, P., *Mol. Cell. Biol. 2*:161 (1982); Gubler, U., and Hoffman, B.J., *Gene 25*:263 (1983); D'Alessio, J.M., *et al.*, *FOCUS 9*:1 (1987)), in a solution containing 25 mM TRIS-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM B-NAD+, 250 μM each of dATP, dCTP, dGTP and dTTP, 1.2 mM DTT, 65 units/ml DNA ligase, 250 units/ml DNA polymerase I and 13 units/ml RNase H.

During the final 30 min of the two-hour second strand cDNA synthesis reaction, streptavidin paramagnetic beads were prepared. Briefly, paramagnetic beads (Life Technologies, Inc.) were resuspended and 150 μl of bead suspension was placed into a microcentrifuge tube for each reaction. The tubes were the placed into a Magna-Sep Magnetic particle Separator (magnet) for two minutes, and supernatant removed by aspiration. The beads were then washed by adding 100 μl of TE buffer (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA) to each tube, resuspending beads, and removing supernatant after two minutes as described above. Following washing, the beads were resuspended in 160 μl of Binding Buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl) and held at 25°C until use in isolating cDNA.

After incubating the second strand cDNA synthesis reaction mixtures with T4 DNA polymerase, the tubes were placed on ice and the reaction terminated by the addition to each tube of 10 μl of 0.5 M EDTA. The biotinylated cDNA molecules were then isolated by contacting the solution with the streptavidin-coupled paramagnetic beads. Briefly, 160 μl of the beads prepared as described above were added to the cDNA reaction mixture tubes, and the tubes gently mixed and incubated for 60 minutes at room temperature. Tubes were then inserted into the magnet for two minutes, after which supernatants were removed and discarded. The beads were then washed by gentle resuspension with 100 μl of wash buffer (10 mM TRIS-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl), followed by re-insertion into the magnet. After two minutes, supernatants were removed and discarded and the washing step repeated. Following the second wash, beads were resuspended in 100 μl of wash buffer, transferred into fresh tubes, and washed twice as above (with five minute exposures to the magnet).

Following the second five-minute wash, supernatant was removed and discarded and cDNA molecules were removed from the beads by incubation with *Not*I. Briefly, 50 µl of *Not*I solution (41 µl of autoclaved distilled water, 5 µl of REact 3 buffer (500 mM TRIS-HCl (pH 8.0), 100 mM $MgCl_2$, 1 M NaCl) and 4 µl of *Not* I) were added to each reaction tube and tubes mixed by gentle pipetting. Tubes were incubated for two hours at 37°C, then inserted into the magnet for two minutes. Supernatants containing the cDNA molecules were withdrawn into a fresh tube, and the beads gently resuspended in 20 µl of TE buffer, re-inserted into the magnet for two minutes, and supernatants from this wash combined with those containing the cDNA molecules from above. To each tube containing pooled supernatants, 70 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the tubes vortexed thoroughly and centrifuged at room temperature for five minutes at 14,000 x *g*. Following centrifugation, 65 µl of the upper, aqueous layer were removed from each tube and transferred into fresh microcentrifuge tubes, and 32 µl of 7.5 M ammonium acetate, 1 µl (20 µg) of Glycogen and 250 µl of cold (-20°C) absolute ethanol were added to each tube. Tubes were then mixed and stored on dry ice or at -70°C for 15 minutes, then centrifuged for 30 minutes at 14,000 x *g* at 4°C. Supernatants were removed and discarded, 100 µl of 70% ethanol were added to the pellets and the tubes were centrifuged for two minutes at 14,000 x *g* at room temperature. Supernatants were removed and discarded, and the pellets were dried in a speed-vac and then dissolved in TE buffer (10 µl for 50-200 ng of input mRNA, or 100 µl for 200-5000 ng of input mRNA). Final cDNA yields were determined by Cerenkov counting.

Example 2: ***Vector Ligation of cDNA and Introduction into Host Cells***

From 10 to 50 ng of the cDNA was ligated into a vector (*e.g.*, pCMVSPORT) and this ligation introduced into *E. coli* by transformation as described in the SUPERSCRIPT Plasmid System manual (Life Technologies, Inc.), except the cloning vector was pre-digested with *Not*I and *Sma*I. In one such ligation, 50 ng of vector was ligated to the cDNA in a 1.5 ml microcentrifuge tube with 4 µl of 5X T4 DNA ligase buffer (250 mM TRIS-

HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG-8000) and 1 μl of T4 ligase (1 unit) at 4°C for 16 hours.

Example 3: ***cDNA Yield Comparisons***

To examine the efficiency and yield of cDNA synthesis by the methods of the invention, cDNA was produced as described above and the amounts produced were compared to those obtained using an alternative commercially available system (SUPERSCRIPT Plasmid System; Life Technologies, Inc., Rockville, Maryland). Briefly, after introducing the pCMV·SPORT-cDNA ligations into MAX EFFICIENCY DH5α™ and ELECTROMAX® DH10B cells, the cells were plated onto ampicillin-containing plates to determine transformation efficiencies. The cDNA inserts were sized by using the SP6 and T7 promoter primers and 40 cycles of PCR on 48 randomly chosen colonies for each experiment.

Table 1 shows a comparison of the cDNA yields obtained by the methods of the present invention to those obtained using the SuperScript Plasmid System.

Table 1. **Comparison of the Invention to the SUPERSCRIPT Plasmid System.**

System Tested	Input mRNA per reaction (ng)	Yield of cDNA (ng)	Transformants per ligation (MAX EFFICIENCY DH5α™)	Avg. Insert Size, basepairs (Range)
Present Invention	1000	117	1.6×10^4	1210 (580-2040)
	5000	619	2.5×10^4	1030 (220-1810)
SUPER-SCRIPT Plasmid System	1000	27	1.8×10^4	840 (450-1400)
	5000	231	2.0×10^4	1280 (240-2080)

These results demonstrate that the present invention produces about three- to four-fold greater yields of cDNA than the SUPERSCRIPT Plasmid System. Furthermore, the present invention demonstrates approximately equivalent transformation efficiencies and average insert sizes to those obtained with the SUPERSCRIPT Plasmid System. Thus, the present invention provides methods for the rapid and efficient production of full-length cDNA molecules without the use of time-consuming and yield-reducing cDNA size fractionation steps.

Example 4: ***Production and Isolation of cDNA Using Varying Amounts of Input mRNA***

Having demonstrated that the methods of the invention produce cDNA rapidly and efficiently, the efficacy of the invention in producing cDNA from varying amounts of input mRNA was examined. In these studies, the amount of input mRNA was varied from 5 ng to 1 μg and the cDNA yield, transformation efficiency and average insert size determined as above. Results are shown in Table 2.

Table 2. **Yield of cDNA Using Different Amounts of Input mRNA.**

Input mRNA per reaction (ng)	**Yield of cDNA (ng)**	**Transformants per ligation (ELECTROMAX® DH10B)**	**Avg. Insert Size, basepairs (Range)**
5	2	2.7×10^5	600 (200-2000)
50	11	5.1×10^6	650 (280-1600)
200	55	8.0×10^6	930 (340-2200)
1000	389	7.5×10^6	1300 (150-2900)

These results demonstrate that the present invention is capable of producing large cDNA libraries (*i.e.*, $>10^5$ clones) from as little as 5 ng of input mRNA. Previously, PCR (a process that biases the cDNA library) was the only method that would have enabled the production of cDNA libraries from this small amount of RNA. Together with those above, these results indicate that the invention is capable of rapidly and efficiently producing high-quality, full-length cDNA molecules from varying quantities of input mRNA, including those that show a low level of expression and thus represent only a small fraction of the polyA+ or total RNA pools."

In pertinent part, the disclosure of United States provisional application serial number 60/122,395, filed March 2, 1999, reads as follows:

"The present invention alleviates, prevents, reduces or substantially reduces internal priming thereby providing improvements in cDNA and cDNA library construction. Accordingly, the present invention greatly facilitates gene discovery by providing cDNA libraries containing a greater percentage of full-length genes.

The present invention therefore relates to synthesizing a cDNA molecule or molecules from an mRNA template or population of mRNA templates under conditions sufficient to increase the total amount of cDNA produced, increase the length of the cDNA molecules produced, and/or increase the amount or percentage of full-length cDNA molecules produced. In accordance with the invention, any conditions which inhibit, prevent, reduce or substantially reduce internal priming may be used. Such conditions preferably include but are not limited to optimizing primer concentrations, optimizing reaction temperatures and/or optimizing primer length or specificity. Such result may also be accomplished in accordance with the invention by optimizing the reverse transcription reaction, preferably by inhibiting or preventing reverse transcription until optimum or desired reaction conditions are achieved.

Conventional methods for constructing cDNA libraries use a molar ratio of oligo(dT) primer/mRNA template of 15:1 for first strand cDNA synthesis. The use of such excess amounts of oligo(dT) primer allows internal priming of one or more primers to one or more of the mRNA templates in the reaction. According to a preferred aspect of the present invention, the amount of oligo(dT) primer is reduced for synthesis of first strand cDNA to inhibit, prevent, reduce or substantially reduce internal priming. Preferred molar ratios of primer to template range from about 12:1; 10:1; 9:1; 8:1; 7:1; 6:1; 5:1; 4:1; 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; 1:5; 1:6; 1:7; 1:8; 1:9; 1:10 and 1:12. Preferably, molar ratios of primer (e.g., oligo(dT)) to template (e.g., mRNA) range from about 5:1 to about 1:20, although lower molar ratios of primer to template may be used in accordance with

the invention. Specifically, molar ratios of primer to template may be below about 1:10; 1:15; 1:20; 1:25; 1:50; 1:75; and 1:100. Preferably, ranges of molar ratios are below about 5:1; 4:1; 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; and 1:5. Most preferably, ratios of primer to template range from about 10:1 to 1:10; 5:1 to 1:10; 4:1 to 1:10; 3:1 to 1:10; 2.5:1 to 1:10; 2:1 to 1:10; 1.5:1 to 1:10; and 1:1 to 1:10. The optimum ratios of primer to template may vary depending on the primer, mRNA, reverse transcription enzyme and reaction conditions (annealing temperature, buffering salts, etc.). The desired primer to template ratios can be readily determined by one skilled in the art.

In conventional methods of cDNA library construction, annealing or hybridizing primer to template is not carried out at a temperature which prevents, inhibits, reduces or substantially reduces internal priming. Typically, the mixture (e.g., mRNA and oligo(dT) primer) is chilled on ice after denaturation or heating. This process typically causes annealing or hybridization of the primer to internal sites. According to a preferred aspect of the present invention, the temperature during the annealing or hybridization between the primer and the template is maintained so that internal priming is inhibited, prevented, reduced or substantially reduced. In accordance with the invention, such a result is accomplished by carrying out primer annealing or hybridization at higher temperatures. Such conditions may also reduce the formation of mRNA secondary structures during cDNA synthesis. Preferably, temperatures for annealing or hybridizing primers to the templates range from about 10°C to about 90°C; more preferably about 10°C to about 80°C; still more preferably about 20°C to about 75°C; more preferably about 25°C to about 75°C; still more preferably about 30°C to about 65°C; still more preferably about 37°C to about 60°C; still more preferably about 40°C to about 60°C; still more preferably about 45°C to about 60°C; still more preferably about 45°C to about 55°C; and most preferably about 45°C to about 65°C. The temperature used may vary depending on the type and amount of primer and template and depending on the temperature optimum of the reverse transcription enzyme. The optimum temperature or temperature ranges can be readily determined by one skilled in the art.

Conventional methods for cDNA synthesis typically requires the use of oligo(dT) primers of a particular length (12-18 bases or more).

Such primer length, however, lowers specificity of the primer thereby allowing internal priming. Thus, the invention also relates to increasing specificity of the primers to prevent, inhibit, reduce or substantially reduce internal priming. In a preferred aspect, primer specificity is increased by increasing the length of the primer. Thus, for cDNA synthesis, longer oligo(dT) primers may be used in accordance with the invention. Preferably, primer length ranges from about 20 to about 100 bases, about 20 to about 75 bases, about 20 to about 60 bases, and about 20 to about 50 bases; more preferably about 20 to about 45 bases; more preferably about 20 to about 40 bases; and most preferably about 25 to about 35 bases. In a preferred aspect, the length of the primers are greater than 19 bases; more preferably greater than about 20 bases; more preferably greater than about 25 bases; and still more preferably greater than about 30 bases. Such primer lengths refer to the length of the primers which anneal or hybridize to the template. Optimum length and content (nucleotide sequence) of the primers may vary depending on the type of template, the desired reaction conditions, and the reverse transcription enzyme. In accordance with the invention, additional sequences and/or modified nucleotides may be included in the primers of the invention. For example, additional sequences (which do not necessarily anneal or hybridize to the template) may be included in the primers of the invention to assist in cDNA synthesis including sequences comprising one or more restriction endonuclease sites, one or more derivative nucleotides (e.g., hapten containing nucleotides such as biotinylated nucleotides), and the like. The type and length of the primers used in accordance with the invention can be readily determined by one or more skilled in the art.

Conventional cDNA synthesis methods do not control or vary activity of the reverse transcription enzyme to optimize the reverse transcription reaction. In accordance with the invention, the activity of the reverse transcriptase is preferably controlled to start synthesis at a desired time in the reaction. In a preferred aspect, reverse transcriptase activity is inhibited or prevented until optimum or desired reaction conditions are achieved. Such a result is accomplished in accordance with the invention

by the use of inhibitors (such as antibodies or antibody fragments) which inhibit reverse transcriptase activity. Such reverse transcriptase inhibitors prevent or inhibit reverse transcriptase activity at low temperatures such that internal priming is prevented, inhibited, reduced or substantially reduced. In accordance with the invention, such inhibitors preferably prevent reverse transcriptase activity below 35°C, below 40°C, below 45°C, below 50°C, below 55°C, below 60°C, below 65°C, below 70°C, below 75°C, below 80°C, below 85°C and below 90°C. Depending on the thermostability of the enzyme having reverse transcriptase activity, the inhibitor may be designed to inhibit activity of the enzyme at a point at or near the temperature optimum for the enzyme of interest. Preferably, the inhibitor is inactivated at a temperature below or near the temperature optimum of the enzyme used, thereby allowing reverse transcription to take place. Thus, the invention generally relates to the use of reverse transcriptase inhibitors in cDNA synthesis. The type and amount of inhibitor may vary depending on the type and amount of reverse transcription enzyme and depending on the reaction conditions to be used. The type of inhibitor and conditions used with such inhibitor can be readily determined by one of ordinary skill in the art.

In accordance with the invention, any one or a combination of the above improvements to cDNA synthesis may be used. Using any one or a combination of these improvements provides for improved first strand cDNA synthesis (e.g., more total cDNA, larger cDNA and/or more full-length cDNA). In accordance with the invention, the first strand cDNA molecules may be used as templates to make one or more double stranded nucleic acid molecules (e.g., double strand cDNA molecules) by incubating one or more of the first strand cDNA molecules produced by the methods of the invention under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the first strand cDNA molecules. Conditions for making double stranded nucleic acid molecules preferably include incubation with one or more components consisting of one or more DNA polymerases, one or more nucleotides, one or more buffering salts, and one or more primers. In another aspect of the invention, such conditions are modified to provide

an increase in the total amount of double stranded cDNA produced, an increase in the length or size of the double stranded cDNA molecule produced, and/or an increase in percentage full-length double stranded cDNA molecule produced. Preferably, such conditions relate to optimization of ribonuclease (RNase) digestion after first strand cDNA synthesis. During first strand cDNA synthesis, if a full-length cDNA molecule complementary to the mRNA template is not made, a single stranded mRNA containing the cap structure will be present at the 5' end of the mRNA of the mRNA/cDNA hybrid. If a full-length cDNA is produced, a double stranded mRNA/cDNA hybrid is produced with no single stranded mRNA present. Preferably, such digestion conditions are optimized so that the single stranded mRNA of the mRNA/cDNA double stranded molecules formed during first strand cDNA synthesis is subject to RNase digestion. In this manner, cap structure from mRNA/cDNA hybrids which are not full-length are removed while full-length mRNA/cDNA hybrids will retain the cap structure. Thus, cap capture can be used to select for full-length molecules and select against molecules which are not fulllength. In a preferred aspect, the conditions are such that the single stranded mRNA of the mRNA/cDNA hybrid is digested or degraded while the mRNA of the double stranded mRNA/cDNA hybrid is not degraded or not substantially degraded. Thus, such RNase digestion is conducted under conditions such that second strand synthesis is not substantially adversely affected. That is, second strand synthesis in accordance with the invention produces larger double stranded cDNA molecules compared to conventional techniques. Conventional RNase I conditions typically range from 25 u/μg to 40 u/μg mRNA at 37°C and RNase A conditions typically are 1000 ng/μg mRNA at 37°C. Using conventional RNase digestion, the average size of double stranded cDNA molecules produced is about 200 bases. According to the present invention the average size of double stranded cDNA molecules produced is preferably greater than about 300 bases, greater than about 400 bases, greater than about 500 bases, greater than about 600 bases, greater than about 700 bases, greater than about 800 bases, greater than about 900 bases, greater than about 1 kilobase, greater than about 1.5 kilobases, and greater than about 2 kilobases. In one

embodiment of the invention, the concentration of the ribonuclease, the type of ribonuclease and reaction conditions are optimized to improve double stranded cDNA synthesis in accordance with the invention. Preferred ribonucleases for use in ribonuclease digestions include ribonuclease A (RNase A) and/or ribonuclease I (RNase I). Generally, lower temperatures (about 4°C to about 50°C) and higher salt concentrations (about 5 mM to about 5 M) will assist in inhibiting or controlling RNase digestion in accordance with the invention. Salts used may include sodium chloride, potassium, chloride, magnesium chloride, sodium acetate etc. Additionally, lowering RNase amounts or concentrations may be used to accomplish the desired result. Such concentrations for RNase A may range from about 0.001 ng/μg mRNA to about 500 ng/μg of mRNA and for RNase I may range from about 0.001 u/μg mRNA to about 500 u/μg mRNA. The incubation temperature, RNase concentration and salt concentration may be readily determined by one skilled in the art. In a preferred aspect, concentration of the RNase A include ranges from 0.1 ng/μg mRNA to 10 ng/μg mRNA in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C. Alternatively, the concentration of the RNase A can include ranges from 0.1 ng/μg mRNA to 500 ng/μg mRNA in 10 mM Tris, pH 7.5 buffer containing 250 mM NaCl at 25°C for 30 minutes. Preferably, concentration of the RNase I used ranges from 0.1 unit/μg mRNA to 1.0 unit/μgmRNA in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 200 mM sodium acetate at 37°C. Alternatively, the concentration of the RNase I can be used at ranges from 1.0 unit/μg mRNA to 100 units/μg mRNA in the same buffer at 25°C for 30 minutes.

In another aspect, the invention relates to capture or binding of the cap structure (e.g., m^7GpppN) of the mRNA before, during or after first strand cDNA synthesis. Thus, the invention relates to selection of mRNA (before first strand synthesis) or mRNA/cDNA hybrids (after or during first strand synthesis) which have the cap structure in carrying out the methods of the invention. Such selection or capture may be accomplished with any cap binding molecule such as eIF4E, eIF4E peptides, eIF4E peptide fragments (see WO 98/08865) and antibodies or antibody fragments specific for cap structure. In a preferred aspect, selection of the

cap structure is accomplished after first strand synthesis. More preferably, such cap capture occurs after ribonuclease digestion in accordance with the methods of the invention. For example, mRNA/cDNA hybrids subjected to ribonuclease digestion are captured and then used for second strand cDNA synthesis according to the invention.

Thus, the present invention is generally directed to methods of synthesizing nucleic acid molecules. The present invention is more specifically directed to methods of making one or more nucleic acid molecules, especially cDNA molecules or cDNA libraries, comprising mixing one or more nucleic acid templates (preferably mRNA, poly A RNA or a population of mRNA molecules) with at least one polypeptide having reverse transcriptase activity, and incubating the mixture under conditions sufficient to make one or more first nucleic acid molecules (e.g., first strand cDNA) complementary to all or a portion of the one or more nucleic acid templates. In accordance with the invention, such conditions provide for an increased total amount of nucleic acid molecule (cDNA) produced, compared to conventional procedures which do not employ the improved modifications or conditions of the invention. The invention also provides for an increase of length or average size of the nucleic acid molecules (cDNA) produced and/or an increase in the percentage or amount of full-length nucleic acid molecules (cDNA) produced, compared to conventional procedures which do not employ the improved modifications or conditions of the invention. Determining the amount, length and full-length content of the cDNA produced can be determined by conventional techniques well known in the art and as described herein. The percentage or average percentages of full-length cDNA in cDNA libraries produced in accordance with the invention are preferably above about 15%, more preferably above about 20%, more preferably above about 25%, more preferably above about 30%, more preferably above about 40%, more preferably above about 50%, more preferably above about 60%, more preferably above about 70%, more preferably above about 80% and most preferably above about 90%. Such full-length percentages are preferably determined by random selection of a portion of the clones of the cDNA library of interest (e.g., 100 to 1000

clones), sequencing the clones and comparing the sequences to known sequence data bases.

In preferred aspects of the invention, the improved results of the invention are preferably accomplished by one or a combination of modifications to the conditions for nucleic acid or cDNA synthesis. Such conditions preferably include modifications for improving first strand cDNA synthesis and/or improving second strand cDNA synthesis.

In a preferred aspect, the invention specifically relates to methods of making one or more double stranded cDNA molecules comprising incubating one or more mRNA molecules (preferably a population of mRNA molecules) with one or more primers of the invention at temperatures and primer concentrations to prevent, inhibit, reduce or substantially reduce internal priming prior to or during first strand cDNA synthesis. Such reaction is preferably conducted in the presence of one or more inhibitors of reverse transcriptase activity in accordance with the invention. Ribonuclease digestion is preferably conducted before second strand cDNA synthesis and at ribonuclease concentrations sufficient to increase the length, amount and/or size of double stranded cDNA molecules produced during second strand synthesis. In accordance with the invention, cap capture is preferably accomplished during or after the ribonuclease digestion.

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The invention also relates to compositions for use in the invention or made while carrying out the methods of the invention. Such compositions may comprise at least one primer (e.g., oligo(dT) or derivatives thereof) and at least one template in a sample or reaction mixture in amounts or ratios in accordance with the invention. Such composition may further comprise one or more polypeptides having reverse transcriptase activity, one or more reverse transcription inhibitors (e.g., anti-RT antibodies or fragments thereof), one or more nucleotides, one or more cap binding molecules (e.g., anti-cap antibodies for fragments thereof), one or more buffering salts and the like. Such

compositions may also be maintained at a temperature to avoid internal priming in accordance with the invention.

The compositions of the invention may also comprise amounts of ribonuclease in accordance with the invention. Such compositions may further comprise at least one component selected from one or more mRNA/cDNA hybrids, one or more nucleotides, one or more polypeptides having reverse transcriptase activity, one or more buffering salts, one or more cap binding molecules (e.g., anti-cap antibodies or fragments thereof) and the like.

The invention also relates to one or more antibodies (monoclonal and polyclonal) and fragments thereof for use in the methods, compositions and kits of the invention. Such antibodies, include anti-cap and/or anti-RT antibodies and antibody fragments.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in the art in view of the following drawings and description of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions:

In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Internal priming as used herein refers to hybridization or annealing of one or more primers at one or more sites within one or more mRNA molecules other than at the poly A tail located at the 3' termini of the mRNA molecule.

Library as used herein refers to a set of nucleic acid molecules (circular or linear) which is representative of all or a portion or significant portion of the DNA content of an organism (a "genomic library"), or a set of nucleic acid molecules representative of all or a portion or significant portion of the expressed genes (a "cDNA library") in a cell, tissue, organ

or organism. Such libraries may or may not be contained in one or more vectors.

Vector as used herein refers to a plasmid, cosmid, phagemid or phage DNA or other DNA molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be inserted in order to bring about its replication and cloning. The vector may further contain one or more markers suitable for use in the identification of cells transformed with the vector. Markers, for example, include but are not limited to tetracycline resistance or ampicillin resistance. Such vectors may also contain one or more recombination sites, one or more termination sites, one or more origins of replication, and the like.

Primer as used herein refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a DNA molecule. Preferred primers for use in the invention include oligo(dT) primers or derivatives or variants thereof.

Oligonucleotide as used herein refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3' position of the deoxyribose or ribose of one nucleotide and the 5' position of the deoxyribose or ribose of the adjacent nucleotide.

Template as used herein refers to double-stranded or single-stranded nucleic acid molecules which are to be amplified, synthesized or sequenced. In the case of a double-stranded molecules, denaturation of its strands to form a first and a second strand is preferably performed before these molecules may be amplified, synthesized or sequenced, or the double stranded molecule may be used directly as a template. For single stranded templates, a primer, complementary to a portion of the template is hybridized or annealed under appropriate conditions and one or more

polymerases or reverse transcriptases may then synthesize a nucleic acid molecule complementary to all or a portion of said template. The newly synthesized molecules, according to the invention, may be equal or shorter in length than the original template.

Incorporating as used herein means becoming a part of a DNA and/or RNA molecule or primer.

Amplification as used herein refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA and/or RNA molecule or primer thereby forming a new molecule complementary to a template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

Nucleotide as used herein refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes ribonucleoside triphosphate ATP, UTP, CTG, GTP and deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, [αS]dATP, 7-deaza-dGTP, 7-deaza-dATP, and biotinylated or haptenylated nucleotides. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Hybridization or **annealing** as used herein refers to base pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA) to give a double-stranded molecule. As used herein, two nucleic acid

molecules may be hybridized or annealed, although the base pairing is not completely complementary. Accordingly, mismatched bases do not prevent hybridization or annealing of two nucleic acid molecules provided that appropriate conditions, well known in the art, are used. In the present invention, the term hybridization or annealing preferably refers to hybridization of one or more primers (e.g., oligo(dT) or derivatives thereof) to one or more templates (e.g., mRNA).

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Polypeptides having reverse transcriptase activity for use in the invention may be obtained commercially, for example from Life Technologies, Inc. (Rockville, Maryland), Pharmacia (Piscataway, New Jersey), Sigma (Saint Louis, Missouri) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Alternatively, polypeptides having reverse transcriptase activity may be isolated from their natural viral or bacterial sources according to standard procedures for isolating and purifying natural proteins that are well-known to one of ordinary skill in the art (see, e.g., Houts, G.E., *et al., J. Virol. 29*:517 (1979)). In addition, the polypeptides having reverse transcriptase activity may be prepared by recombinant DNA techniques that are familiar to one of ordinary skill in the art (see, e.g., Kotewicz, M.L., *et al., Nucl. Acids Res.* 16:265 (1988); Soltis, D.A., and Skalka, A.M., *Proc. Natl. Acad. Sci. USA 85*:3372-3376 (1988)).

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A variety of DNA polymerases are useful in accordance with the present invention. Such polymerases include, but are not limited to, *Thermus thermophilus (Tth)* DNA polymerase, *Thermus aquaticus (Taq)* DNA polymerase, *Thermotoga neapolitana (Tne)* DNA polymerase, *Thermotoga maritima (Tma)* DNA polymerase, *Thermococcus litoralis (Tli* or VENT™) DNA polymerase, *Pyrococcus furiosis (Pfu)* DNA polymerase, DEEPVENT™ DNA polymerase, *Pyrococcus woosii (Pwo)* DNA polymerase, *Bacillus sterothermophilus (Bst)* DNA polymerase, *Bacillus caldophilus (Bca)* DNA polymerase, *Sulfolobus acidocaldarius (Sac)* DNA polymerase, *Thermoplasma*

acidophilum (Tac) DNA polymerase, *Thermus flavus (Tfl/Tub)* DNA polymerase, *Thermus ruber (Tru)* DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum (Mth)* DNA polymerase, *Mycobacterium* spp. DNA polymerase *(Mtb, Mlep),* and mutants, variants and derivatives thereof.

DNA polymerases used in accordance with the invention may be any enzyme that can synthesize a DNA molecule from a nucleic acid template, typically in the 5' to 3' direction. Such polymerases may be mesophilic or thermophilic. Mesophilic polymerases include T4 DNA polymerase, T5 DNA polymerase, T7 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III, DNA polymerase I and the like. Thermostable DNA polymerases include *Taq*, *Tne, Tma, Pfu*, VENT™, DEEPVENT™, *Tth* and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene* 112:29-35 (1992); Lawyer, F.C., *et* al., PCR *Meth. Appl. 2*:275-287 (1993); Flaman, J.-M., *et al., Nucl. Acids Res. 22(15):*3259-3260 (1994)).

DNA polymerases for use in the invention may be obtained commercially, for example from Life Technologies, Inc. (Rockville, Maryland), Perkin-Elmer (Branchburg, New Jersey), New England BioLabs (Beverly, Massachusetts) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana).

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The invention may be used in conjunction with any methods of cDNA synthesis that are well-known in the art (see, e.g., Gubler, U., and Hoffman, B.J., *Gene 25*:263-269 (1983); Krug, M.S., and Berger, S.L., *Meth. Enzymol.* 152:316-325 (1987); Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989); PCT US98/19948; and WO 98/51699) to produce cDNA molecules or libraries. Other methods of cDNA synthesis which may advantageously use the present invention will be readily apparent to one of ordinary skill in the art.

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The cDNA molecules or libraries produced by the invention may also be further manipulated by standard molecular biology techniques such as two hybrid analysis, cDNA normalization, sequencing and amplification. More particularly, the methods of the invention and the cDNA molecules or libraries produced by such methods may be used in combination with RT-PCR and 5' RACE technology (Life Technologies, Inc.) and differential display.

A variety of inhibitors and binding molecules are suitable for use in the present methods. Included among these inhibitors or binding molecules are antibodies that bind to the above-described polypeptides having reverse transcriptase activity (such as anti-RT antibodies including anti-AMV RT antibodies, anti-M-MLV RT antibodies or anti-RSV RT antibodies) or to cap structure (e.g., anti-cap antibodies), and fragments thereof (such as Fab or $F(ab')_2$ fragments). Such antibodies may be polyclonal or monoclonal, and may be prepared in a variety of species according to methods that are well-known in the art. See, for instance, Sutcliffe, J.G., *et al., Science 219*:660-666 (1983); Wilson *et al., Cell 37*: 767 (1984); and Bittle, F.J., *et al., J. Gen. Virol. 66*:2347-2354 (1985). Antibodies specific for any of the above-described reverse transcriptases or cap structures can be raised against the intact polymerase polypeptide or cap structures or one or more fragments thereof. These polypeptides or cap structures or fragments thereof may be presented together with a carrier protein (e.g., albumin) to an animal system (such as rabbit or mouse) or, if they are long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) may be used interchangeably with the terms "polyclonal antibody" or "monoclonal antibody" (mAb), except in specific contexts as described below. These terms, as used herein, are meant to include intact molecules as well as antibody fragments (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of specifically binding to a polypeptide having reverse transcriptase activity (such as a DNA polymerase or a reverse transcriptase) or cap structures or portions thereof.

The antibodies used in the methods of the present invention may be polyclonal or monoclonal, and may be prepared by any of a variety of methods (see, e.g., U.S. Patent No. 5,587,287). For example, polyclonal antibodies may be made by immunizing an animal with one or more polypeptides having reverse transcriptase activity or cap structures or portions thereof according to standard techniques (see, e.g., Harlow, E., and Lane, D., *Antibodies: A Laboratory Manual,* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (1988); Kaufman, P.B., *et al.,* In: *Handbook of Molecular and Cellular Methods in Biology and Medicine,* Boca Raton, Florida: CRC Press, pp. 468-469 (1995)). Alternatively, monoclonal antibodies (or fragments thereof) to be used in the present methods may be prepared using hybridoma technology that is well-known in the art (Köhler *et al., Nature 256*:495 (1975); Köhler *et al., Eur. J. Immunol.* 6:511 (1976); Köhler *et al., Eur. J. Immunol. 6*:292 (1976); Hammerling *et al.,* In: *Monoclonal Antibodies and T-Cell Hybridomas,* New York: Elsevier, pp. 563-681 (1981); Kaufman, P.B., *et al.,* In: *Handbook of Molecular and Cellular Methods in Biology and Medicine,* Boca Raton, Florida: CRC Press, pp. 444-467 (1995)).

It will be appreciated that Fab, $F(ab')_2$ and other fragments of the above described antibodies may be used in the methods described herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). Antibody fragments may also be produced through the application of recombinant DNA technology or through synthetic chemistry.

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Examples:

[Example 5]:

Comparison of first strand cDNA synthesis with varying ratios of oligo (dT) primer/mRNA.

This example compares first strand cDNA synthesis of the MAP4 gene with various ratios of oligo dT primer/starting mRNA. All components are available from Life Technologies, Inc., Rockville, Maryland, unless specified otherwise.

The master mix for Superscript II reverse transcriptase (SS II RT) was prepared as specified in [Table 3] below.

[Table 3]

Component	µl	µl
5X SSII RT buffer	4	28
0.1 M DTT	2	14
10 mM dNTP	1	7
α-^{32}P_dCTP	0.5	3.5
Water	1.5	10.5
Total volume	**9**	**63**

The master mix for ThermoScriptTM II RT (TS RT) (AMV RT αH-βH+) (see WO 98/47921) was prepared as specified in [Table 4] below.

[Table 4]

Component	µl	µl
10X TS II buffer*	2	38
0.1 M DTT	2	38
10 mM dNTP	2	38
(α-^{32}P dCTP	0.5	9.5
RNase OUT (40	1	19
Water	1.5	28.5
Total volume	**9**	**171**

*10X TS II buffer comprises 50 mM Tris-HC1 (pH 8.4), 750 mM KCI, and 75 mM $MgCl_2$. The master annealing mix was prepared by adding a 5 Kb MAP4 mRNA, oligo$(dT)_{25-30}$ and water to 5 tubes in the amounts specified in [Table 5] below.

[Table 5]

Ratio of oligo (dT)/mRNA	1:1	2.5:1	5:1	10:1	50:1
Component	Volume (µl)	Volume	Volume (µl)	Volume	Volume
MAP4 mRNA (1 µg/µ1)	5	5	5	5	5
Oligo$(dT)_{25-30}$ (10 ng/µ1)	2.6	6.5	13.1	-	-
Oligo$(dT)_{25-30}$(100 ng/µ1)	-	-	-	2.6	13.1
Water	42.4	38.5	31.9	42.4	31.9
Total volume	**50**	**50**	**50**	**50**	**50**

The mixture was heated at 70°C for 10 minutes and then chilled on ice for 5 minutes.

Synthesis of first strand cDNA was done by adding 9 µl of the appropriate reverse transcriptase master mix, 10 µl of the master annealing mix and 1 µl of either SS II RT (200 units/ µl) or TS II RT (15 units/ µl) for a total volume of 20 µl as summarized in [Table 6] below.

[Table 6]

Tube	Reverse Transcriptase	Temperature	Ratio of olio $(dT)_{25-30}$/ mRNA
1	SS II	45°C	1
2			2.5
3			5
4			10
5			50
1	TS II RT	45°C	1
2			2.5
3			5
4			10
5			50
6	TS II RT	50°C	1
7			2.5
8			5
9			10
10			50
11	TS II RT	55°C	1
12			2.5
13			5
14			10
15			50

The reactions were incubated for 1 hour at 45°C for SS II RT and at 45, 50 or 55°C for TS II RT. The tubes were placed on ice to complete the reaction. 18 µl first stand cDNA of the reaction tube was precipitated and re-suspended in

10 µl of water. 5 µl of the first strand cDNA was mixed with 5 µl of standard loading buffer (60 mM NaOH, 4 mM EDTA, 0.1% bromophenol blue), and loaded onto 1.4% alkaline agarose gel for analysis. * * *

* * * *

The results show that by reducing the molar ratio of oligo(dT) primer/mRNA (preferably to 1:1) internal priming with reverse transcriptase was almost entirely eliminated.

[Example 6]: Comparison of first strand cDNA synthesis under standard and hot start conditions

This experiment was designed to compare first strand cDNA synthesis of the MAP4 gene with standard reaction and hot start conditions.

The annealing mix was prepared by mixing 1 µg of MAP4 mRNA and biotinylated *Not* I oligo$(dT)_{25}$ primer ($(Biotin)_4$ GACTAGTTCTAGAT CGCGAGCGG CCGCCCTTTTT TTTTTTTTTTTT TTTTTTTT [(SEQ ID NO: 13)]; see WO 98/51699) in the desired molar ratio of oligo (dT)/mRNA of 0:1, 1:1 or 15:1 in thin-walled PCR tubes and bringing the volume up to 10 µl with water. If several tubes are identical, they may be made in one batch and aliquotted accordingly. The annealing mix was kept on ice.

The master mix for Superscript II reverse transcriptase (SS II RT) was prepared as specified in [Table 7] below.

[Table 7]

Component	µl	µl
5X SSII RT buffer	4	28
0.1 M DTT	2	14
10 mM dNTP	1	7
α-^{32}PdCTP	0.5	3.5
SSII RT (200 u/µl)	1	7
Water	1.5	10.5
Total volume	**10**	**70**

The SS II RT master mix was then divided into two equal aliquots, one for processing with standard reaction temperatures (batch 1) and one for processing with hot start reaction temperatures (batch

2). To allow for condensation, an additional 10% volume of water was added to batch 2. All mixes were kept on ice.

Synthesis of first strand cDNA was begun by briefly spinning tubes containing annealing mix to collect droplets, placing the tubes in a thermocycler and then heating them to 70°C for 10 minutes. After this 10 minute cycle at 70°C, the tubes of annealing mix for batch 1 were immediately removed to ice. The tubes of annealing mix for batch 2 were allowed to cool to 45°C in the thermocycler while the batch 2 master mix was placed in the thermocycler and incubated at 45°C for 5 minutes. After the 5 minute incubation, 11 µl of the master mix for batch 2 was added to each batch 2 annealing tube and mixed with a pipette 2 times. Care was taken not to spin the tubes to avoid lowering the temperature.

10 µl of the master mix for batch 1 was added to each batch 1 annealing tube. The batch 1 tubes were lightly vortexed and briefly centrifuged to collect condensation droplets. The batch 1 tubes were then returned to the thermocycler and the tubes from both batch 1 and 2 were incubated at 45°C for one hour.

5 µl of the first strand cDNA from each tube was mixed with 5 µl of standard loading buffer (60 mM NaOH, 4 mM EDTA, 0.1% bromophenol blue) and loaded onto 1.4% alkaline agarose gel for analysis. * * *

* * * *

First strand cDNA was also synthesized with TS II RT using 15 units of TS II RT per µg mRNA using a biotinylated oligo(dT)/mRNA ratio of 1:1 and 15:1. The same protocol described above was followed, except that the temperature was varied to 50°C.

* * * *

The results indicated that by dropping the reaction temperature to the reverse transcriptase reaction temperature after denaturation of the primer and mRNA mixture, the reaction was started directly and internal priming was avoided entirely.

[Example 7]: Synthesis of double strand cDNA by controlling the reaction temperature and the concentration of salt and RNase

This example describes the synthesis of double stranded cDNA by controlling the reaction temperature and the concentration of salt and different ribonuclease (RNases) during the treatment of the cDNA/mRNA hybrids after first strand cDNA synthesis.

First strand cDNA was synthesized as described above in [Example 6] and digested with either RNase I or RNase A as further described below.

RNase I digestion of first strand cDNA was done by re-suspending the first strand cDNA in 180 µl of water and 20 µl of 10X RNase I buffer (100 mM Tris-HCI (pH 7.5), 50 mM EDTA, 2 M sodium acetate). 2.5 units of RNase I (1 unit/µg mRNA) were added and the mixture was mixed well. The RNase I digestion mixture was incubated at 25°C for 30 minutes and extracted with phenol/chloroform once. The supernatant was precipitated with 1 µlof glycogen, 100 µ1 of ammonium acetate and 800 µ1 of ethanol.

RNase A digestion of first strand cDNA was done by re-suspending the first strand cDNA in 200 µl of digestion buffer (10 mM Tris-HCI (pH 7.5), 250 mM NaCl). 12.5 ng of RNase A (5 ng/µg mRNA) were added and the mixture was mixed well. The RNase A digestion mixture was incubated at 25°C for 30 minutes and extracted with phenol/chloroform once. The supernatant was precipitated with 1 µl of glycogen, 100 µl of ammonium acetate and 800 µ1 of ethanol.

[Example 8]: Enrichment of the full-length cDNA clones with cap-binding, proteins

This example describes enrichment of full-length cDNA clones with the capbinding protein eIF4E.

cDNA was prepared by precipitating the RNase I treated first strand cDNA described in [Example 7] above and washing with 70% ethanol. The resulting pellet was dried at room temperature for 5 minutes, and re-suspended in 210 µ1 of 10 mM KPO_4, 100 mM KCl, 2 mM EDTA, 6 mM DTT and 5% glycerol. The cDNA was stored on ice.

eIF4E glutathione sepharose 4B beads were prepared by first mixing glutathione sepharose 4B beads (Pharmacia, Sweden) well. To prepare eIF4E beads, a recombinant host cell expressing GST tagged eIF4E protein (the eIF4E gene was cloned into a GST fusion vector to create a N-terminal GST-eIF4E fusion gene) was grown and the fusion protein was purified by standard techniques. Thus, the invention also relates to recombinant host cells expressing eIF4E protein (particuarly as fusion proteins), to vectors comprising the genes expressing such proteins or fusion proteins and to the recombinant proteins or fusion proteins produced. In the present invention any tag can be used (e.g., His Tag, GST tag, HA tag, *Trx* tag, etc.). Such tags may be positioned at the carboxy and/or N-terminal region of the eIF4E gene.

The GST-eIF4E fusion protein was complexed with sepharose 4B beads by glutathione coupling using gluthionine sepharose 4B beads (Pharmacia Biotech) following the manufacturers protocols. 200 µl of the beads were transferred to a 1.5 ml microcentrifuge tube, centrifuged for 1 second, and 75 µl of supernatant was removed. The beads were washed twice with 1 ml of reaction buffer (10 mM KPO_4, 100 mM KCl, 2 mM EDTA, 6mM DTT and 5% glycerol), and re-suspended in 258 µl of reaction buffer, followed by the addition of 42 µl (18 pmoles/µl) of eIF4E protein (600 pmoles/10 µl beads). The mixture was mixed on a head to head roller at 4°C for 30 minutes. The mixture was then centrifuged for 1 second, and the supernatant was removed. The beads were washed twice with 1 ml of reaction buffer and once with 1 ml of 25 µg/ml yeast tRNA in reaction buffer. 20 µl of reaction buffer and 5 µg of yeast tRNA were then added to the beads. 200 µl of RNase I treated cDNA was added to the beads, and the content was mixed on a roller at room temperature for 1 hour. After 1 hour, the mixture was centrifuged for 1 second, and the supernatant was removed. The beads were washed twice with 1 ml of reaction buffer and once with 1 ml of 500 µM GDP in reaction buffer. The cDNA was eluted twice with 250 µl of 500 µM GDP in reaction buffer. The eluted solutions were pooled and centrifuged for 1 minute to remove the beads. The eluted cDNA was extracted twice with an equal volume of phenol/chloroform. The cDNA was divided into two tubes and

precipitated with 1 μl of glycogen, 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of ethanol.

[Example 9]: Evaluation of the cDNA library

To evaluate the quality of the cDNA libraries constructed with the abovedescribed full-length methods, the MAP4 gene (5-6 kb) and other genes was selected as the target genes. MAP4 and other cDNA clones were isolated from libraries constructed by standard methods well-known in the art (see SuperScript™ Plasmid Manual, Life Technologies, Inc.) and the above-described full-length methods with 3' and 5' GeneTrapper cDNA Positive Selection System (Life Technologies, Inc., Rockville, Maryland). The positive clones were size analyzed by PCR. [Tables 8 and 9] below summarizes the results of the enrichment of full-length cDNA clones in human fibroblast cDNA libraries constructed with methods well-known in the art (control) and the full-length methods described above (full-length method).

[Table 8]

Gene	% full-length with 5' GeneTrapper		% full-length with 3' GeneTrapper	
	control*	full-length method	control*	full-length method
MAP4 (5-6 kb)	12.8	90.3	6.25	37.5

*The control library was constructed with SS II RT using known methods

[Table 9]

Gene name	Full-length of gene (Kb)	% Full-length by 5'
MAP4 (Microtubule-associated protein 4)	5/6	90.3
β-Adaptin*	3.8/5.7	90.0
TR (Transferrin receptor)	5.0	45.0

PTK (Protein tyrosine Kinase)	3.0	84.4
RPA (DNA Replication protein A)	1.4	98.0

* There are two members of the genes, 3.8 kb and 5.7 kb in the family.

These results show that the full-length methods described above yielded >90% full-length cDNA clones with the 5' GeneTrapper system, compared to <13% using standard methods. Furthermore, the above-described full-length methods yielded >37% full-length clones with the 3' GeneTrapper system, as compared to <7% using standard methods.

[Example 10]: First strand cDNA synthesis, RNase I digestion and eIF-4E capture

All conditions and parameters described above in [Examples 6, 7 (RNase I) and 8] were followed, except for the following: 4 reactions of 10 μg of human fibroblast cytoplasmic mRNA were used per reaction (see WO 98/45311); the biotinylated primer-adapter $(Biotin)_4$-$GACTAGTTCTAGATCGCGAGCGGCCGCCC(T)_{25}$ [(SEQ ID NO: 13)] was used at a 1:1 primer/mRNA molar ratio; TS II RT was used at 50°C; and SS II RT was used at 45°C. [Table 10] below summarizes the first strand cDNA and eIF-4E capture results.

[Example 11]: Second strand cDNA synthesis

Second strand cDNA was synthesized by first dissolving each of the four reaction pellets obtained in [Example 10] above in 104 μl of DEPC-treated water and then adding the following reagents to each reaction:

4 μl of 5X First Strand Buffer*

30 μl of 5X Second Strand Buffer*

2 μl of 0.1 M DTT

4 μl of 10 mM dNTPs

1 µl of *E. coli* DNA ligase (10 units/µl)

1 µl of *E. coli* RNAse H (2 units/µl)

4 µl of *E. coli* DNA polymerase (10 units/µl)

* see SuperScript Plasmid System manual (Life Technologies, Inc., Rockville, Maryland)

These reactions mixtures were then incubated for 2 hours at 16°C. 2 µl of T4 DNA polymerase (5 units/µl) was added and incubation at 16°C was continued for 5 more minutes.

[Example 12]: Streptavidin Bead Preparation

During the last 30 minutes of the 2 hour second strand reaction described in [Example 11] above, streptavidin paramagnetic beads were prepared as follows. Streptavidin paramagnetic beads (Seradyn) were gently mixed by pipetting until the beads were completely re-suspended. 150 µl of the mixed beads were transferred to the bottom of a microcentrifuge tube for each reaction. The tubes were inserted into a Magna-Sep Magnetic Particle Separator (Life Technologies, Inc., Rockville, Maryland) (the magnet) and let sit for 2 minutes. While the tubes were in the magnet, the supernatant was removed by pipetting and 100 µl of TE buffer (10 mM Tris-HCI (pH 7.5), 1 mM EDTA) was immediately added to the beads.

The tubes were then removed from the magnet and the beads were gently resuspended by finger tapping or vortexing at the lowest setting. The tubes were reinserted into the magnet. After 2 minutes, the supernatant was removed, the beads were re-suspended in 160 µl of binding buffer (10 mM Tris-HCI (pH 7.5), 1 mM EDTA, 1 M NaCl) and the tubes were placed into a microcentrifuge tube rack.

[Example 13]: Capture of the double-stranded cDNA library

After incubating the second strand reaction with T4 DNA polymerase as described in [Example 12] above, the reaction mixtures were placed on ice and 10 µl of 0.5 M EDTA was added. Then the cDNA

library was captured according to the following procedure (see generally WO 98/51699). The paramagnetic beads prepared according to Example 8 were transferred to the second strand reaction mixture tubes and gently mixed by pipetting and the suspension was incubated for 60 minutes at room temperature. The tubes were then inserted into the magnet. After 2 minutes, the supernatant was removed and discarded.

100 µl of wash buffer (10 mM Tris-HCI (pH 7.5), 1 mM EDTA, 500 mM NaCl) was added to the beads, the beads were re-suspended by finger tapping or gently vortexing at the lowest setting and the tubes were re-inserted into the magnet for 2 minutes. The supernatant was removed and discarded. This washing step was repeated one more time and then 100 µl of wash buffer was added to the beads. The tubes were then again inserted into the magnet for 5 minutes.

[Example 14]: *Not* I Digestion

After the 5 minute incubation described in the last step of [Example 13], the supernatant was removed and discarded from the paramagnetic beads and 41 µl of autoclaved, distilled water, 5 µl of REact 3 buffer, 4 µl of *Not* I was added and the beads were mixed well by pipetting. The reaction was then incubated for 2 hours at 37°C. The tubes were then inserted into the magnet for 2 minutes and the supernatant containing the cDNA library was transferred to fresh tubes.

50 µl of phenol: chloroform:isoamyl alcohol (25:24:1) was added to the supernatant, the solution was vortexed thoroughly, and then centrifuged at room temperature for 5 minutes at 14,000 x g. 45 µl of the upper, aqueous layer was carefully removed and transferred to fresh microcentrifuge tubes. 23 µl of 7.5 M ammonium acetate, 1 µl of glycogen (20 µg) and 172 µl of ethanol (-20°C) was added. The solution was mixed well and stored on dry ice (or -70°C freezer) for 15 min.

The ethanol solution was then centrifuged at 4°C for 30 minutes at 14,000 x g. The supernatant was carefully removed from the small pellets. 100 µl of 70% ethanol was added and the tubes were centrifuged at room

temperature for 2 minutes at 14,000 x g. The ethanol was removed and the pellets were dried in a speed-vac for 2 minutes or until dry. The pellets were then dissolved in 20 µl of TE buffer (10 mM Tris-HC1 (pH 7.5), 0.1 mM EDTA). The final yield of cDNA was determined by the Cerenkov counts (see [Table 10] below).

[Table 10]

Reverse Transcriptase	standard (S) or varied (V) temperature	% Incorporation (ng of eDNA)	Amount of cDNA after eIF-4E capture
TS II RT	S	27% (2,720 ng)	512 ng
TS II RT	V (hot start)	26% (2,640 ng)	473 ng
SS II RT	S	46% (4,560 ng)	306 ng
SS II RT	V (hot start)	47% (4,730 ng)	363 ng

[Example 15]: Ligation of cDNA to the vector and introduction into *E.coli*

From 10 to 30 ng of the un-fractionated or size fractionated (≥1.5 kb by low melting gel electrophoresis) cDNA was ligated into a vector pCMVSPORT 6 (Life Technologies, Inc.). This ligation was introduced into *E. coli* by electroporation as described in the SuperScript Plasmid System manual (Life Technologies, Inc., Rockville, Maryland), except that the cloning vector was pre-digested with *Not* I and *Eco* RV.

Sequence analysis of randomly selected clones from the cDNA library constructed (304 clones) were analyzed by 5' and 3' sequencing to determine the total percentage of full-length random clones in the cDNA library. Sequences were compared for homology with GeneBank sequences. The results are summarized in [Table 11] below. Based on the results, approximately 68% of the random clones were full-length (including known full-length clones and unknown full-length clones). Thus, approximately 17% unknown full-length clones were obtained from the human fibroblast cytoplasmic mRNA library.

[Table 11]

	Number of Clones	Percentage
Total Sequences	304	73.3%
Sequences with Homology	223	51%
Full-Length_Clones	114	17%
Potentially Full-Length	39	17%
Partial Clones	70	31%

[Example 16:] RNAse Assay

First strand cDNA was treated with RNase A at 1000 ng/μg mRNA in TE buffer (10 mM Tris-HCI (pH 7.5), 1 mM EDTA) and RNase 125 to 40 u/μg mRNA in TEN (10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8.0), 200 mM Sodium Acetate) at ~37°C essentially as described in [Example 7]. However, this treatment with large amounts of RNase at elevated temperatures resulted in libraries containing very small average cDNA insert size (about 200 bp). Therefore, a second strand cDNA assay was developed to determine the optimal amount of RNase needed.

First strand cDNA (radioactively labeled and non-radioactively labeled) was synthesized using HeLa mRNA at 500 ng of RNA/reaction. The first strand cDNA was precipitated with ethanol and dissolved in DEPC-treated water. The cold first strand cDNA was added to RNase buffer with different amounts of RNase. After incubation for 30 minutes at 25°C, the treated cDNA was extracted with phenol:chloroform and precipitated with ethanol. The treated cDNA was dissolved in DEPC-treated water, a second strand cDNA reaction was performed with ^{32}PdCTP plus and minus RNase H. The reaction was extracted with phenol:chloroform and precipitated with ethanol. Equal amounts of cpm was electrophoresed into a 1.4% alkaline-agarose gel.

* * * *

These gel analysis demonstrated that a concentration of 1.25 ng of RNAse A * * * or 0.5 units of RNAse I * * * may be optimal to use with 500 ng of starting mRNA."

Other methods of cDNA synthesis which may advantageously use the present invention will be readily apparent to one of ordinary skill in the art.

Having obtained cDNA molecules or libraries according to the present methods, these cDNAs may be isolated for further analysis or manipulation. Detailed methodologies for purification of cDNAs are taught in the GENETRAPPER™ manual (Life Technologies, Inc.; Rockville, Maryland), which is incorporated herein by reference in its entirety, although alternative

standard techniques of cDNA isolation known in the art may be used *(see, e.g.,* Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989)).

In other aspects of the invention, the invention may be used in methods for amplifying nucleic acid molecules. Nucleic acid amplification methods according to this aspect of the invention may be one-step (*e.g.,* one-step RT-PCR) or two-step (*e.g.*, two-step RT-PCR) reactions. According to the invention, one-step RT-PCR type reactions may be accomplished in one tube thereby lowering the possibility of contamination. Such one-step reactions comprise (a) mixing a nucleic acid template (*e.g.*, mRNA) with one or more polypeptides having reverse transcriptase activity and with one or more DNA polymerases and (b) incubating the mixture under conditions sufficient to amplify a nucleic acid molecule complementary to all or a portion of the template. Alternatively, amplification may be accomplished by mixing a template with one or more polypeptides having reverse transcriptase activity (and optionally having DNA polymerase activity). Incubating such a reaction mixture under appropriate conditions allows amplification of a nucleic acid molecule complementary to all or a portion of the template. Such amplification may be accomplished by the reverse transcriptase activity alone or in combination with the DNA polymerase activity. Two-step RT-PCR reactions may be accomplished in two separate steps. Such a method comprises (a) mixing a nucleic acid template (e.g., mRNA) with one or more reverse transcriptases, (b) incubating the mixture under conditions sufficient to make a nucleic acid molecule (e.g., a DNA molecule) complementary to all or a portion of the template, (c) mixing the nucleic acid molecule with one or more DNA polymerases and (d) incubating the mixture of step (c) under conditions sufficient to amplify the nucleic acid molecule. For amplification of long nucleic acid molecules (*i.e.*, greater than about 3-5 Kb in length), a combination of DNA polymerases may be used, such as one DNA polymerase having 3' exonuclease activity and another DNA polymerase being substantially reduced in 3' exonuclease activity. An alternative two-step procedure comprises the use of one or more polypeptides having reverse transcriptase activity and DNA polymerase activity (*e.g., Tth, Tma or Tne*

DNA polymerases and the like) rather than separate addition of a reverse transcriptase and a DNA polymerase.

Amplification methods which may be used in accordance with the present invention include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822).

Kits

In another embodiment, the present invention may be assembled into kits for use in reverse transcription or amplification of a nucleic acid molecule. Kits according to this aspect of the invention comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like. The kits of the invention may comprise one or more components selected from one or more reverse transcriptases, one or more DNA polymerases, one or more suitable buffers, one or more nucleotides, one or more solid supports (particularly FTA® or derivatives or variants thereof) and/or one or more primers.

In a specific aspect of the invention, the reverse transcription and amplification kits may comprise one or more components (in mixtures or separately) including one or more, polypeptides having reverse transcriptase activity, one or more supports, one or more nucleotides needed for synthesis of a nucleic acid molecule, and/or one or more primers (*e.g.*, oligo(dT) for reverse transcription). Such reverse transcription and amplification kits may further comprise one or more DNA polymerases. Preferred polypeptides having reverse transcriptase activity, DNA polymerases, nucleotides, primers and other components suitable for use in the reverse transcription and amplification kits of the invention include those described above. The kits encompassed by this aspect of the present invention may further comprise additional reagents and compounds necessary for carrying out standard nucleic acid reverse transcription or amplification protocols. Such polypeptides having reverse transcriptase activity, DNA polymerases, nucleotides, primers, and

additional reagents, components or compounds may be contained in one or more containers, and may be contained in such containers in a mixture of two or more of the above-noted components or may be contained in the kits of the invention in separate containers.

Use of Nucleic Acid Molecules

The nucleic acid molecules or cDNA libraries prepared by the methods of the present invention may be further characterized, for example by cloning and sequencing (*i.e.*, determining the nucleotide sequence of the nucleic acid molecule), or by the sequencing methods *(see, e.g.,* U.S. Patent Nos. 4,962,022 and 5,498,523, which are directed to methods of DNA sequencing). Alternatively, these nucleic acid molecules may be used for RPA, northern blots or attachment to chips for the manufacture of various materials in industrial processes, such as hybridization probes by methods that are well-known in the art. Production of hybridization probes from cDNAs will, for example, provide the ability for those in the medical field to examine a patient's cells or tissues for the presence of a particular genetic marker such as a marker of cancer, of an infectious or genetic disease, or a marker of embryonic development. Furthermore, such hybridization probes can be used to isolate DNA fragments from genomic DNA or cDNA libraries prepared from a different cell, tissue or organism for further characterization.

The nucleic acid molecules of the present invention may also be used to prepare compositions for use in recombinant DNA methodologies. Accordingly, the present invention relates to recombinant vectors which comprise the cDNA or amplified nucleic acid molecules of the present invention, to host cells which are genetically engineered with the recombinant vectors, to methods for the production of a recombinant polypeptide using these vectors and host cells, and to recombinant polypeptides produced using these methods.

Recombinant vectors may be produced according to this aspect of the invention by inserting, using methods that are well-known in the art, one or more of the cDNA molecules or amplified nucleic acid molecules prepared according to the present methods into a vector. The vector used in this aspect of the invention may be, for example, a phage or a plasmid, and is preferably a

plasmid. Preferred are vectors comprising *cis*-acting control regions to the nucleic acid encoding the polypeptide of interest. Appropriate *trans*-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression (and are therefore termed "expression vectors"), which may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker such as a tetracycline or ampicillin resistance gene for culturing in a bacterial host cell. Prior to insertion into such an expression vector, the cDNA or amplified nucleic acid molecules of the invention should be operatively linked to an appropriate promoter, such as the phage lambda P_L promoter, the E. *col i lac, trp* and *tac* promoters. Other suitable promoters will be known to the skilled artisan.

Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNHl6a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV-SPORTI and Gateway™ Vectors, available from Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

The invention also provides methods of producing a recombinant host cell comprising the cDNA molecules, amplified nucleic acid molecules or recombinant vectors of the invention, as well as host cells produced by such methods. Representative host cells (prokaryotic or eukaryotic) that may be produced according to the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells. Preferred bacterial host cells include *Escherichia coli* cells (most particularly *E. coli* strains DHIOB and

Stbl2, which are available commercially (Life Technologies, Inc; Rockville, Maryland)), *Bacillus subtilis* cells, *Bacillus megaterium* cells, *Streptomyces* spp. cells, *Erwinia* spp. cells, *Klebsiella spp.* cells and *Salmonella typhimurium* cells. Preferred animal host cells include insect cells (most particularly *Spodoptera frugiperda Sf9* and *Sf21* cells and *Trichoplusa* High-Five cells) and mammalian cells (most particularly CHO, COS, VERO, BHK and human cells). Such host cells may be prepared by well-known transformation, electroporation or transfection techniques that will be familiar to one of ordinary skill in the art.

In addition, the invention provides methods for producing a recombinant polypeptide, and polypeptides produced by these methods. According to this aspect of the invention, a recombinant polypeptide may be produced by culturing any of the above recombinant host cells under conditions favoring production of a polypeptide therefrom, and isolation of the polypeptide. Methods for culturing recombinant host cells, and for production and isolation of polypeptides therefrom, are well-known to one of ordinary skill in the art.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

All reagents and media were from Life Technologies, Inc, Rockville, MD unless otherwise stated.

EXAMPLE 17: Preparation and Storage of Nucleic Acids on Solid Supports

Cell culture.

HeLa cells were grown in suspension in S-MEM with 10% heat-inactivated horse serum and 4 mM glutamine and BHK-21 cells were grown in

monolayer as described (7) and suspensions were prepared by trypsinization followed by washing and resuspension in Dulbecco's PBS (containing Ca^{2+} and Mg^{2+}), at the appropriate cell density. Resuspended cells were spotted on FTA® GeneCards using an adjustable pipettor and similar control samples were vialed and quick frozen in a dry-ice ethanol bath and stored at -70°C.

Preparation and storage of samples. 20 µl of blood and 5 µlof HeLa cell suspension (1 x 10^7 cells/ml) were spotted directly on FTA® GeneCards, allowed to air dry for up to 2 h, and stored at room temperature, 4°C, -20°C, or -70°C in sealed foil packages containing desiccant. Gene Guard Swabs containing buccal cells were applied onto FTA® GeneCards, allowed to air dry for up to 2 hours and stored at room temperature in sealed foil packages with desiccant. Plants were grown in soil and leaf samples were obtained. Plant leaf samples were pressed onto FTA® GeneCards using a nitrogen-driven press (17.5 psi) and treated as described above.

EXAMPLE 18: Isolation of Poly(A^+)RNA Directly from Cells on FTA® Paper

20-50 µl of a BHK-21 cell suspension (4.25 x 10^7/ml) was spotted directly onto FTA® GeneCards and stored at -70°C as described above or placed in tube, frozen in dry ice ethanol and placed at -70°C. For RNA isolation, the entire spot was cut into small pieces using a razor blade and added to 750 µl of sterile water followed by incubation at room temperature for 15 min with frequent vortexing. To remove the filter pieces, the eluate was passed through a shredder microfuge tube (Qiagen, CA) and the poly(A+) RNA isolated by selection with oligonucleotide(dT). Typical yields from these samples were 300 ng mRNA/ 2x10^6 cells. Total RNA from BHK-cells was isolated using TRIzol™ Reagent according to the manufacturer's directions and poly(A^+)RNA was isolated from these samples by selection with oligo(dT).

EXAMPLE 19: Northern Blot Analysis of RNA

Total RNA and Poly(A^+)RNA were subjected to electrophoresis in a 1.5%, 1X MOPS, 30% formaldehyde agarose gel as described (8) followed by transfer to a nylon membrane. The blot was baked at 80° C for 1 h followed by

prehybridization as described (8). ^{32}P-labeled b-actin probe was prepared using the RadPrime kit (Life Technologies, Inc) and was adjusted to a final concentration of 5 X 10^6 cpm/ml hybridization buffer. Hybridization was performed as described (8) for 16 h at 42° C . The blot was washed 3 x 5 min with 2x SSC containing 0.1% SDS at room temperature and 2 x 30 min with 0.25x SSC containing 0.1% SDS at 65°C. The blot was then placed in plastic wrap and exposed to X-ray film.

The results of the Northern blot analysis are shown in Figure 1. Total RNA (lane 1) was isolated from BHK-21 cells using TRIzol Reagent. Poly(A+)RNA was isolated from the total RNA (lanes 2-3) or directly from BHK-21 cells applied to the FTA® GeneCard (lanes 4-5) as described above. The number of cells used was 2.5 x 10^6 (lanes 2 and 4) and 4 x 10^6 (lanes 1, 3 and 5).

The quality and intensity of the 2.2-kb signal from the FTA® archived samples is directly comparable to that of RNA isolated from vialed BHK-21 cells by traditional means. Based on these results, it appears that the integrity of poly(A+)RNA from mammalian cell samples spotted onto FTA® GeneCards is maintained. However, is has been found that after application of mammalian cells onto FTA® paper, the samples must be placed at temperatures $\leq$ -20°C for long term storage (greater than 1 month). RNA integrity in samples stored at room temperature or 4°C for extended periods was sub-optimal compared to controls. Genomic DNA contained in FTA®-archived samples stored at room temperature for up to 7.5 years has been shown to be intact (9), which is quite different from our observations with RNA.

EXAMPLE 20: Amplification of Nucleic Acids

PCR of genomic DNA. Using a HARRIS MICRO-PUNCH®, 2-mm punches were removed from the center of the biological sample spot, placed in a 1.5 ml microfuge tube and processed by washing 3 x 5 min with FTA® Purification Reagent (Life Technologies Inc.) at room temperature followed by 2 x 5 min washes with TE (10 mm Tris-HCl pH 8.0, and 0.1 mM EDTA) at room temperature. Each punch was processed individually and then transferred to a thin-walled amplification tube. Amplification was performed

by using PLATINUM® *Taq* High Fidelity DNA polymerase (IU), in 1X PLATINUM® *Taq* High Fidelity PCR Buffer, 200 mM dNTPs, 200 nM primers, and 2 mM $MgSO_4$. The sequences of the primers used for the amplification reactions are shown in Table 12.

Table 12. Primer Sequences used. (SEQ ID NOS 1-12, respectively in order of appearance)

Target (Human)	Primer Sequences	Product size
b-globin	Sense: 5'-CTGCAGTCCCAGGCTATTCAGG-3' Antisense: 5'-AGACTTGGACCATGACGGTGAT-3'	1.3 kb
b-globin	Sense: 5'-CTGCTGAAAGAGATGCGGTGG-3' Antisense: 5'-TCTTCCCAAAATGCCCTGAGT-3'	3.19 kb
Cysteine protease (plant)	Sense: , 5'-TCGCCGATCTGACTAATGAGGAG-3' Antisense: 5'-ATGCGCTTCATTGCCTTCACTCC-3'	1.05 kb
Replication protein A	Sense: 5'-CAAGATGTGGAACAGTGGATTC-3' Antisense: 5'-CATCTATCTTGATGTTGTAACAAGC-3'	1.08 kb
b-actin	Sense: 5'-CCTCGCCTTTGCCGATCC-3' Antisense: 5'-GGATCTTCATGAGGTAGTCAGTC-3'	0.626 kb
Clathrin-like protein	Sense: 5'-CCCAGTGACAGGAGGAGACCATA-3' Antisense: 5'-ATCCTGTGCTTTTTCTGTGGGAC-3'	5.76 kb

RT-PCR. Using a HARRIS MICRO-PUNCH®, 2-mm punches were transferred to 1.5 ml low-binding RNase-free DNAse-free tubes (Marsh Biomedical) containing 400 µl of RNA processing buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 400 - 800 U/ml RNASEOUT® and 2 mM DTT) and incubated on ice for 25 min with vortexing every 5 min.. In some experiments, the processing buffer also contained 250 µg/ml glycogen to facilitate subsequent precipitation of the RNA. Unlike genomic DNA, RNA elutes from the filter punches during this incubation. RT-PCR was done either directly using the processing buffer eluate as substrate or using RNA precipitated from the eluate. The RNA was precipitated by addition of salt (0.1 volumes of 3 M sodium acetate, or 0.5 volumes of 7.5 M ammonium acetate) and 0.5 volumes of ice cold 100% isopropanol. The samples were placed at -20°C overnight, spun down at 12,000 rpm in the microfuge, washed with 75% ethanol (ice-cold) and allowed to air dry. RNA pellets were resuspended in 50 µlor 100 µlof sterile TE. Synthesis of first strand cDNA was performed using SUPERSCRIPT® It RNase H- RT (Life Technologies, Inc) in a final volume of 50 µl at 50°C. Amplification reactions (50µl) contained ≤ 10 µl of the cDNA reaction and the following: 1X Amplification Buffer, 1.8 MM $MgSO_4$, 200 nM primers, 200 mM of each dNTP and 2.5 U of PLATINUM® *Taq* DNA polymerase. For templates larger than 4 kb, 1-2U of PLATINUM® *Taq* DNA Polymerase High was used. Amplification products were analyzed by 1.2% TBE-OR 0.8% TAE agarose gel electrophoresis.

The results of the amplification of nucleic acids stored on solid supports are shown in Figures 2-4. Figure 2 shows the results of the amplification of nucleic acids from HeLa cells. Eluted RNA was precipitated from washes taken from 2-mm punches of HeLa cell samples stored at -20° and -70°C for 1 year as described above. The amplification targets were as follows: Panel A; a 626 bp sequence from b-actin mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 40 cycles of 94°C for 30 s; 60°C for 30 s and 72°C for 1.5 min; forward and reverse primer sequences were 5'CCTCGCCTTTGCCGATCC3' (SEQ ID NO: 9) and 5'GGATCTTCATGAGGTAGTCAGTC3' (SEQ ID NO: 10), respectively. Panel B; a 1.08-kb sequence of RPA (replication protein A)

mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 40 cycles of 94°C for 30 s; 55°C for 30 s and 72°C for 1.5 min; forward and reverse primer sequences were 5'CAAGATGTGGAACAGTGGATTC3' (SEQ ID NO: 7) and 5'CATCTATCTTGATGTTGTAACAAGC3' (SEQ ID NO: 8), respectively. and Panel C: a 5.76-kb sequence of a clathrin-like protein (D21260) mRNA was amplified using the following thermocycling conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 20 s; 60°C for 30 s and 68°C for 7 min; forward and reverse primer sequences were 5'CCCAGTGACAGGAGGAGACCATA3' (SEQ ID NO: 11) and 5'ATCCTGTGCTTTTTCTGTGGGAC3' (SEQ ID NO: 12), respectively. For Panels A and B, Lanes 1-3 and 4-6 are from samples stored at -20°C and -70°C, respectively subsequent to sample application onto FTA® GeneCards, whereas lane 7 is a negative control where SUPERSCRIPT II RT was omitted from the RT reaction. Lanes labeled M are a 1 kb ladder size markers. For Panel C, lanes l, positive control, HeLa RNA, Lanes-2 and 3 are from samples stored at -70°C subsequent to sample application onto FTA® GeneCards, whereas lane 4 is the negative control.

Figure 3 shows the results of the amplification of nucleic acids from plant cells. RNA was eluted from 2-mm punches of the leaf samples from potato plants as described in above and 5 µl of the RNA eluate was added to each 50 ml RT reaction. The amplification target was a 1.05-kb sequence from a 1.8-kb cysteine protease (AJ003137) mRNA using the primers shown in Table 1. Thermocycling conditions were: 94°C for 1 min, followed by 40 cycles of 94°C for 30 s; 60°C for 30 s; and 72°C for 2 min. Lanes 1-3 and 4-7 are from samples stored at -20C and -70C, respectively subsequent to application on FTA® GeneCards, whereas lane 8 is a positive control where 50 ng of potato leaf RNA was added to the RT reaction.

The dependence of RT-PCR signal on amount of biological sample stored on card was examined and the results are shown in Figure 4. 5 µl samples of suspensions of HeLa cells at different cell densities were spotted onto FTA® GeneCards, allowed to air dry for 1-2 hours and then stored at -70°C for over 1 year. 2-mm punches were taken from the samples and treated

as described above. An aliquot of the RNA (1/80th of the total volume of the wash) was used for RT-PCR as described in Methods. The target was a ~1.08 kb amplicon of the RPA (replication protein A gene, (M36951) using the primers indicated in Table 1 and the PCR conditions used in Figure 2. Marker, 1 Kb Plus ladder. Lane 1-3, 25,000 cells; Lane 4-6, 5,000 cells; Lanes 7-9, 500 cells, Lanes 10-12 negative controls where SuperScript™ II RNase H- RT was omitted from the RT reaction.

RNA stability on FTA® GeneCards stored at temperatures ≤ -20°C was examined by performing RT-PCR analysis on different mRNA targets. In processing the FTA® punches, it was observed that unlike genomic DNA, RNA does not remain on the FTA® paper during processing. Virtually all of the RNA elutes into the initial wash, and this eluted cellular RNA can be directly placed into the first strand RT reaction or can be ethanol precipitated from the wash solution and resuspended in sterile water or TE prior to analysis. The results in Figures 2 and 3 demonstrate successful RT-PCR of different mRNA targets from mammalian cells and plant samples, respectively. For the mammalian cell samples, our RT-PCR targets were 626-bp, 1.08-kb and 5.76-kb sequences from b-actin (Panel A), replication protein A (RPA; Panel B) and clathrin-like protein (Panel C) mRNAs, respectively. For the potato leaf plant samples, our RT-PCR target consisted of a 852-bp sequence from the 1756-bp cycteine protease mRNA. Negative controls consisted of reactions where RT was omitted during the initial cDNA synthesis step (not shown for plant samples) and positive controls consisted of the addition of 100 ng of HeLa or 50 ng of plant leaf RNA directly to the initial cDNA synthesis step. It is important to include the negative control since we have observed that trace amounts of genomic DNA also elute from the punch during processing and it is necessary to ascertain that RT-PCR signals are indeed products from RNA and not contaminating genomic DNA. The results in Figures 2 and 3 demonstrate that the desired RNA-specific RT-PCR products were obtained with the FTA® samples stored at -20°C and -70°C and were comparable to the positive controls. We next examined the proportionality of RT-PCR signal obtained versus the number of HeLa cells that were spotted onto the FTA® GeneCard. Such an experiment would reveal the feasibility of using this method to semi-quantitatively measure differential

gene expression in biological samples. HeLa cell suspensions at various densities were prepared and 5 µl aliquots of the various suspensions were identically spotted onto FT A® paper. The relative amount of RT-PCR product obtained was proportional to the number of cells placed onto the FTA® card (Figure 4). These data indicate that at least semi-quantitatively, differences in mRNA levels can be measured by RT-PCR using FTA® Gene Cards.

EXAMPLE 21: cDNA Library Construction from RNA Isolated from Biological Specimens Stored* on *FTA® Paper

Poly(A+)RNA was directly isolated from 2.25 x 10^6 BHK-21 cells stored on FTA® paper as described above except that the biotinylated oligonucleotide(dT) had special adapter sequences necessary for library construction. The primer includes a *Not* I recognition site and has the sequence $(Biotin)_4$ GACTAGTTCTAGAT CGCGAGCGG CCGCCCTTTTT TTTTTTTTTTTT TTTTTTTT (SEQ ID NO: 13); (see WO 98/51699 and United States application serial number 09/076,115). As a positive control, poly(A+) RNA was isolated total RNA prepared by TRIzol reagent from the same number of cells. Double-stranded cDNA was made and cloned into plasmid vectors as described in WO 98/51699 and United States application serial number 09/076,115. The number of primary clones obtained from the poly(A+)RNA was the same whether the mRNA was isolated directly from FTA® or from TRIzol-purified total RNA. The average insert size of the libraries was determined by colony PCR using primers to the plasmid vector. The average insert size for the FTA®-derived material was greater than that for the library constructed from the positive control poly(A+)RNA, 1000bp vs 600 bp. This indicates that cDNA libraries of good quality can be made from mRNA isolated directly from samples stored on FTA®.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and

that such modifications or changes are intended to be encompassed within the scope of the appended claims.

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SEQUENCE LISTING

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